The original drawing was made by Leonardo da Vinci (1452 - 1519) as an illustration of a paragraph in the well-known book of the Roman architect Vitruvius, dealing with the proportions of the human body (sketchbook MS B, fol 39, recto, Library "Institut de France", Paris). The square was transformed into a block diagram representing the total body transport function $\psi(t)$. Thus a connection was established between the universal artist and scientist da Vinci and the integral science of systems dynamics, which in this dissertation was applied to pharmacokinetics.
A Systems Dynamics Approach
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CLINICAL PHARMACOKINETICS OF NICOTINE, CAFFEINE, AND QUININE

A Systems Dynamics Approach

EEN WETENSCHAPPELIJKE PROEVE
OP HET GEBOED VAN DE
WISKUNDE EN NATUURWETENSCHAPPEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
VOLGENS BESLUIT VAN HET COLLEGE VAN DE CANEN
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"Learning hath gained most
by those books
by which the printers
have lost".

T. Fuller, 1642.

To my parents

To my beloved wife

Together have we
troubled and joyful times
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GENERAL ACCOUNT

The work described in this thesis derives its relevance from the massive human consumption of the three alkaloids studied. Indeed, nicotine, caffeine, and quinine are self-administered extensively by people spread all over this planet, belonging to many cultures and religions, either sex and all social classes. From infancy on, children become habituated to the sight and the smell of adults who smoke and drink coffee, tea or quinine-flavored soft drinks. Sooner or later they will let themselves be enticed into the first trying-out of these tempting habits. In many instances they will indulge in the pharmacological actions for the rest of their lives and so continue the habit.

Western society is infested with all kinds of expressions and manifestations of nicotine, caffeine, and (be it to a less extent) quinine use. It is suggested that nowadays man does not only seek relief in tobacco and coffee from the stresses and distresses of life, but that, as an element of modern society, he or she adopts the habit with the codes and prevailing fashions of that society. Smoking and coffee drinking are now more than a personal indulgence in a psychological and pharmacological gratification; they have acquired the status of a ceremonial gesture with a significance of its own in the complicated social rites.

In this preface we will devote a few words to the side of human nature that leads man to take drugs that affect his mood and behaviour, or merely the taste of the nourishments and beverages that he takes. Although caffeine and nicotine exert pharmacological actions throughout the body, their appreciated stimulant actions mainly involve the central nervous system, these two compounds belong to the former category. Quinine, which is esteemed as a bitter principal in soft drinks, belongs to the latter category. The rate of quinine self-administration by devotees of these drinks typically is small to such an extent that the (untoward) central effects of quinine in general are not felt.

Subsequently, separate sections will be dedicated to each of the studied alkaloids, we will mention their natural sources, tell the history of their past and present use(s), and we will try to extrapolate these data in order to predict the nature and extent of their future uses.
Opinions tend to be sharply divided as to the purpose of life, or, as some like to word it: man's destiny. Profound metaphysical questions of this kind are entirely outside the scope of this thesis, but, whatever the personal leanings may be, it must surely be conceded that human behaviour is partly governed by the desire to avoid or abolish pain, fear, restlessness and other disagreeable sensations. One of the most influential great philosophers of our century, who has dealt with this theme exhaustively, is Sigmund Freud. It is the central point of a doctrine propounded by him, in which he asserts that man is assailed on all sides by influences that disturb his peace of mind in some degree.

Freud dealt with the question as to what man demands of life by formulating the pleasure-pain principle, pointing out that human nature is bent primarily on seeking pleasure and avoiding suffering and dissatisfaction. Apart from its formulation, this was no new psycho-analytical discovery. The early philosophers had already quested for solutions to those difficulties of life which may conveniently be condensed into the words: man's predicament. Such solutions ranged from the unrestricted gratification of all desires to the avoidance of any dissatisfaction.

In 'Das Unbehagen in der Kultur', Freud, referring to the causes of man's predicament, states that we are inadequately equipped to meet the challenge of life, which brings us grief and disappointment and all too often imposes impossible tasks. We cannot face life without palliatives, whether these be distraction and relaxation or luxuries and intoxicants. Man strives for happiness; he wants to attain and retain it, but it will rarely endure for long. Change sometimes brings a real sense of joy, but usually it is transitory.

Freud detects three sources of dissatisfaction: our own bodies, the outside world, and our fellow-men. Our bodies warn us of danger by sending us signals of pain and fear; they have only limited powers of endurance with which to meet a host of onslaughts, direct or potential, upon life and limb. We have learned to control the danger immanent in natural forces to a considerable extent but, paradoxically, that danger has increased in proportion to our growing control of them. The third, and to many the most important, source of unhappiness is the unsatisfactory realisation of human relationships in the family and in society.

Man's attempts to rid himself of his sense of frustration can be divided into two groups. He may either eradicate the cause or, if this is impossible
or too difficult, resort to various mitigating expedients. Whatever one thinks of the cause, life obviously imposes such a heavy burden upon man that he finds it necessary to seek a means of lightening this burden and of turning his environment into an artificial paradise, of transforming 'being' into 'well-being'.

The technique, in the broadest sense of the word, consists in attacking the causes of dissatisfaction. As far as our own bodies are concerned, it amounts to hygiene and medical therapy. To protect ourselves from the forces of nature we have developed a technique in the narrower sense, namely houses to shield us from the elements, roads to facilitate travel and vehicles to minimize physical exertion. It is in removing the difficulties besetting human contacts that we have been slow to progress. The tensions between small and large groups of people, neighbours, colleagues, competitors, social classes, and nations, set up a mental stress which we are still powerless to combat.

Freud believed that, here again, we are up against a bit of indomitable nature, namely our psychical make-up. As far back as 1930 he pointed out that the then prevailing spirit of unrest, unhappiness and fear were largely due to the realization that man now controlled the forces of nature to the extent of easily being able to use them for the extermination of the human race. He then could not have foreseen how amazingly our efficiency in that respect would have increased within a few decades.

Much has been written about the makeshifts resorted to in order to reach a compromise with the sources of disruption referred to. Freud calls the taking of drugs the least refined, but the most effectual method, for, in addition to creating pleasurable sensations, it assures detachment. The subject knows that, whenever required, the drug will afford escape from the pressure of reality. Freud considers many psychic symptoms of great diversity to be a more subtle means of escape than the taking of drugs, mentioning neurosis, religion, phantasy, day-dreaming, humour, and a large number of artistic and cultural activities.

The fact that the means of escape adopted are so numerous and so various may be accounted for by the general human urge to replace the existing world by another. In a literal sense, the quest for another world finds expression in the desire to travel and make voyages of discovery. (During one of these, Columbus discovered the 'New World', as well as a new world of drug indulgence, tobacco.) In this context, scientific research and discoveries are to be classed as originating from the same urge, and sports falls into the same category. Temporary release from the drudgery or troubles of life can be
obtained by active and passive participation in sports. The (ab)use of doping agents may give an extra dimension to the feeling of escape during active sporting, and the commitment of mass vandalism may do this job for the "passive sporter" (1) Cinema and the mass media may legitimately be said to serve the same purpose.

This is not a complete list of disguised attempts to put something else in the place of an unsatisfactory reality, to resolve man’s predicament. We do not want to complete it either. Our main concern in view of the subject of this thesis is with drugs taken for the purpose of rubbing of the rough edges of reality. It is evident from many sources of inquiry that mankind had recourse to these from time immemorial, and probably they will accompany man until eternity.

The most innocent form of pharmacological indulgence is the flavouring of foods and beverages with aromatic "drugs" to improve their taste. Man probably learnt to sweeten his food with honey before he mastered articulate speech. Today the quest for synthetic low-caloric sweetening agents without pertinent side- and aftertastes is an important research topic in flavouring industry. Modern man wishes to enjoy sweetness as eagerly as his Palaeolithic ancestors stealing honeycombs from wild bees. Like the latter tried to prevent being stung, the former tries to elude obesity and caries. Spices and salt have been man's table companions as long as honey and sugar. The history of bitter agents like quinine is less well-documented and illustre, presumably because a bitter taste is experienced as unsavoury by a major part of the population, especially by children. Caffeine also has a pronounced bitter taste, which contributes to the bitterness of coffee and tea and which is masked in cola-flavoured soft-drinks by the addition of large amounts of sweeteners.

Drugs taken for their influence on the central nervous system are less innocent and must be under suspicion, even if their effects are mild at the amounts in which they are usually taken. After all use of such drugs may be dangerous for the user and/or people in his proximity if the drug induces a state of hallucination in which physical perils (e.g., in traffic) are no longer properly recognized (alcohol, opiates), or if the drug may effect/catalyse a state of rage and aggressiveness (alcohol, Rohypnol), or if the drug merely intoxicates if taken in large amounts (caffeine, nicotine, alcohol). We will try to formulate some of the 'merits' that qualify a given substance as a desirable tranquilliser or exhilarant.

Primarily, of course, it must produce the required effect. It must liberate the excessively inhibited, remove the anxieties from the worrier, and cheer up
the sad At the same time, however, it must be no too injurious, at any rate in moderate doses Man wants to compromise between the euphoria of intoxication and the desire to preserve his health, a compromise which determines his choice from the many drugs and the routes of their administration which nature and human ingenuity have placed at his disposal Experience has shown that the true exhilarants often weigh the scale in favour of intoxication Pure pick-me-ups seldom do any physical harm but, their efficacy as a means of escape being commensurately small, they are felt to be inadequate in acute conditions

Often it is not the soporific effect of drugs of indulgence that counts in the first place Drugs like caffeine and nicotine may intensify perception, facilitate flow of thought and increase one's ability to concentrate on difficult tasks, and thus enhance man's ability to cope with reality, be it for a limited time and maybe at the cost of some dullness after that time Also, these two and other drugs (e.g., alcohol) may promote sociability rather than a desire to flee from society This stimulation of performance and the desire for human contact may be valuable in themselves, though the resulting conduct (in case of alcohol), the adverse health effects and the involuntary co-smoking of fellow-men in our close proximity (in case of smoking) may tip the balance of appreciation in a contrary direction

The universal popularity of any one particular drug depends on its accessibility Formerly, when one had no option but to take what nature afforded locally, the geographical factor was very important At one time the Eskimos took no kind of a drug or an intoxicant at all, for the simple reason that there were none to be had, whereas the Equitorial aboriginals had quite an assortment to choose from Later, when international trading grew and facilitated travel brought the peoples of the world and the continents ever closer, geographical remoteness counted less as a limiting factor The changed attitude of 'civilised' man towards intoxication and its social side-effects, such as body neglect, a repulsive appearance, economic dislocation and aggressiveness, is significant To the primitives, intoxication was of supernatural origin, the strange, to them inexplicable, behaviour which it evoked put the seal of sanctity on it The intoxicated received prophetic visions, spirits took possession of his body and spoke through his mouth (oracle) Thus intoxication offered the supreme means of entering into the presence of those higher powers which, in their view, held the destiny of mankind in their hands

Cost is another factor comparable with geographical accessibility In many cases it is fixed mainly by the authorities, who impose excise duty on some
articles (tobacco, coffee, tea, and alcohol), or even ban them (marihuana, opiates). In the latter case there has always been a black market offering the forbidden drugs at exorbitant prices, burdening society with addicts operating in the criminal circuit to raise the necessary finances.

By weighing the advantages against the disadvantages of certain substances, it might be possible to draw up a set of norms which should, as far as possible, fit within the framework of hygiene. A wise selection and use of the drugs depend fundamentally upon precise knowledge of their effects at various concentrations in blood plasma and other body fluids. Knowledge of the significance of the different ways in which one particular drug is taken is likewise indispensable. For example, it undoubtedly makes all the difference in several respects whether tobacco is chewed, sniffed, or smoked, and, in the latter case, whether it is smoked as cigars or as cigarettes.

The problem approached in this way is a fascinating puzzle. However, it cannot be wholly dealt with within the framework of the present dissertation. From now on we will (have to) put our minds mainly to the one piece of the puzzle we chose to study: the characteristics of the time course of three selected drugs in human body fluids, and the way of their disposal. While concentrating on pharmacokinetics, we deliberately have to leave all other aspects including the social, psychological, philosophical, and preventive-medicinal aspects, of the use of these three and other drugs to our colleagues in these respective fields.

This dissertation contributes to the knowledge of the pharmacokinetics of three of the most frequently enjoyed drugs of our era, and of the main metabolite of two of them. The scope of the present piece of research has been listed briefly in this preface. The natural sources and the utilitive histories of nicotine, caffeine, and quinine now will pass under separate review.

NICOTINE: NATURAL OCCURRENCE, ABUNDANCE, AND ITS HISTORY AS A DRUG OF INDULGENCE

'Fumer, c'est mourir un peu'.
Adriaan Morriën.

Nicotine is considered to be the principal alkaloid in commercial tobaccos which are derived from the dried leaves of the Nicotiana species tabacum and rustica, where it occurs to the extent of 2-8% combined with citric and malic
The nicotine molecule contains a single chiral carbon center, of the two optical isomers, only \((-\)-S-nicotine occurs in \textit{Nicotiana} species. The latter enantiomer is more potent than the \((+)-R\)-enantiomer in stimulating nicotinic receptors.

The botanical genus \textit{Nicotiana}, established by Linnaeus in 1753, belongs to the \textit{Solanaceae} family, also known as the potato or nightshade family, and contains at least 60 species. Morphologically and cytogenetically, there is a direct relationship between South American and Australian-South Pacific species, whereas North American species, although related to the former, are only indirectly related to the latter. This combined evidence points to South America as the center of current distribution of the genus \textit{Nicotiana} (36 endemic species), and, together with other pertinent data, argues for the origin of the genus in that continent with subsequent dispersal to North America (15 endemic species, 6 of which occur in South America as well) and to Australia and the South Pacific (15 endemic species).

The only species which has never been found growing wild is \textit{Nicotiana tabacum}, which is the species the most widely grown for commercial use. It is an amphidiploid having arisen from hybridization of two South American species \textit{N. sylvestris} and \textit{N. tomentosiformis}. The earlier mentioned species, \textit{N. rustica}, is grown only in limited areas of China, India, and the USSR and plays a minor ever decreasing commercial role.

The many alkaloidal and related materials found in plants of the genus \textit{Nicotiana} comprise nicotine, nornicotine, anabasine, myosmine, anatabine, nicotinic acid, nicotinamide, nicotyrine, 2,3'-dipyridyl, and many others. In most \textit{Nicotiana} species, nornicotine is more abundant than nicotine.

Tobacco holds an unparalleled position among crop plants, while in overall situation the status achieved by the \textit{tabacum} species is quite remarkable. Some of the more noteworthy points are listed below:

- It is one of the very few crops entering the world trade entirely on leaf basis.
- It is the most widely grown commercial non-food plant in the world.
- In many countries it is an instrument of high importance in financial and economic policy.
- Consumption is rather diverse, by way of smoking, inhalation of dust (snuff), and chewing.
- As a habit-forming narcotic, tobacco with fair regularity has been the subject of attacks aiming at curtailing or abandoning its use. However, consumption has marched steadily forward.
tobacco use originally having religious significance, subsequent claims of medicinal benefit have alternated with accusations of a causal role in the etiology of many diseases.

In order to understand how the present mondial status of tobacco use came into existence, we now will devote a few words to its history. Before the coming of the white man, the two species of current economic importance, \textit{N. tobacco} and \textit{N. rustica}, were under cultivation in parts of both Americas and of the West Indies. We will never know how the Indians came to use tobacco. Maybe they burned the spontaneously dried-up old lower leaves of the tobacco plants, the so-called sand-leaves, for cooking purposes or to chase away the mosquitos, and inevitably inhaled bits of the evolving smoke. Anyway they proceeded to cultivate it in the neighbourhood of their settlements. Apparently \textit{N. rustica} was the tobacco of the tribes east of the Mississippi, of a number of those somewhat westward and of the Indians of northern Mexico. Andean Indians did not seem to cultivate \textit{N. tabacum} is commonly referred to as widespread in aboriginal cultivation in eastern South America from Brazil northward, and in Colombia, Central America, most of Mexico and the West Indies. In North America a number of indigenous species of \textit{Nicotiana} have been used by Indian tribes. Of such species perhaps the best known is \textit{N. bigelovii}.

Tobacco was called 'tobaco' in Mexico, 'petum' in certain regions of Brazil, 'uppowoc' in Virginia and 'ziq' by the Mayas. The native Indian population of both Americas used tobacco as a stimulant. It was considered a divine gift, an important part of their religious life was hung on it, and many medicinal qualities were attributed to it. The latter idea was taken over by the Europeans later.

Tobacco was completely absent in the rest of the world. No written tradition, be it in Sanskrit, in Hebrew, or in Egyptian hierglyphics, mentions it, and even Marco Polo who described every event and detail happening during his journey to and in China, kept silent about anything that could pass for tobacco.

The non-availability of tobacco did not prevent the rest of the world from smoking. Other herbs were burned in order to inhale the smoke. According to Herodotus the Scythians possessed a plant, the leaves of which were thrown into the flames. The old Egyptians smoked \textit{majorn}, Plinius prescribed the smoke of coltsfoot as a remedy for coughing, and thyme, mint or cannabis were to be smoked as afrodisiacs.
Two crewmen who accompanied Columbus to the New World were the first Europeans to observe the smoking of tobacco, the 28th of October 1492, on the eastern shore of Juana (Cuba) "The two messengers who had been put ashore to scout the land told that on their way they had met many aboriginals carrying burning torches and also held special herbs in their hands to incense themselves as is the custom among them" (log-book, the 6th of November 1492) Columbus was handed a gift, composed of dried leaves that were considered very valuable by the aboriginals. The leaves and the burning torches (cigars) were called 'tobago'. Bishop Bartholome de las Casas, who accompanied Columbus on his first journey to the New World, wrote about the Indians of Hispaniola (Jamaica) that "they always had a flame at hand, and certain herbs to smoke. These were dried and wrapped in a dry leaf." In 1496, friar Ramon Pane, Columbus' domestic anthropologist, first described the ritual of Tahitian Indians sniffing up tobacco which had been grinded to powder, and in 1499, Amerigo Vespucci observed Indians on Margarita, an island near the Venezuelan coast, to chew tobacco. In 1518, Juan de Grialva during an expedition in Mexico encountered a precursor of the modern cigarette: a small hollow reed-stalk filled with tobacco. In Florida before the very eyes of Ponce de Leon (in 1513) and those of Verrazzano (in 1524) the Indians also gave evidence of mastering pipe smoking.

Columbus' crew returned to Spain with a large stock of Havanas-avant-la-lettre. Because of its supposed curative effects, tobacco was popularized rapidly in Spain. The Portuguese soon obtained the herb from their colony Brazil, the Dutch laid hand on it by plundering Spanish and Portuguese ships. Around 1550, a lot of smoking was to be observed in Spain, Portugal and Holland. Before the end of the 16th century, seamen of the three maritime nations had spread the fame of tobacco and the techniques of consuming it to seaports all around the known part of the world.

Well-known is the story of Jean Nicot de Villemain (1530-1600), French diplomat in Portugal. His queen, Catharina de Medici, suffered from unbearable attacks of migraine, until Jean Nicot sent her a sample of sniffing tobacco (1560). It did the trick. Catharina was given considerable relief, and in this way tobacco started its career in France.

In Italy, cardinal Prospero di Santa-Croce introduced tabacco one year later, and John Hawkins in 1565 did the job for England. Germany became acquainted with tobacco as 'Catharina's herb' in 1570, when the Huguenots took it with them while fleeing from France. A Spanish diplomat introduced it at the Court of Vienna in about that same year.

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However, tobacco had powerful enemies in those early days for all kinds of reasons. The Christian Church associated inhalation of tobacco and other herbs with heathen worship and strongly rejected the use of this 'Satan's herb.' The Spanish Inquisition accused Columbus' crewman Rodrigo de Jerez of being possessed by demons. Pope Urbanus VII threatened to excommunicate every smoker, but his successor already had to tolerate priests smoking during the Service. King Henry VIII held out the prospect of whiplashes to smokers. Elizabeth I tried to confiscate pipes and tobacco boxes. Her successor, James Stuart I, forbade the smoking habit altogether and issued a 6 shilling penalty. In 1618 he accused Sir Walter Raleigh of high treason and let him be executed. Raleigh ascended the scaffold smoking pipe. Many English pipe manufacturers fled to Gouda in Holland when subsequently they were forbidden to provide their pipes with a baked-up sculpture of Raleigh's face.

In the Middle East smokers were hanged with their pipes put cross-wise in their mouth or in their nose. The Persian sjah Abbas ordered smokers' lips to be cut off, when even this horrible sanction was not able to ban the smoking habit, Abbas' successor went as far as spearing smokers. The Russian tsar Michael ordered smokers' noses to be cut off, inveterate smokers were sent to Syberia, and whoever even there got hold of tobacco was decapitated. When in Japan the god-emperor (mikado) altogether interdicted the use of tobacco, his citizens for the first time in many centuries were provoked to exhibit civil disobedience.

Despite all this profane and ecclesiastical opposition the number of tobacco users kept growing steadily ever since Columbus first set foot on American soil. Governments' resistance against smoking rapidly disappeared after the authorities discovered the possibility of lining the Treasury with duties excised on tobacco. The Republic of Venice was the first to do so at the turn of the 15th century.

Cultivation methods for tobacco plants and leaf curing techniques had been improved markedly at the dawn of the industrial era, which brought along machinal cigarette manufacture (1884). The resulting large scaling-up of cigarette production, accompanied by a resulting decrease of the cigarette prices paved the way for the huge mass-consumption of tobacco in the shape of predominantly cigarettes as we know it today. Other factors spreading the use of tobacco and stimulating its consumption rate are:

- large-scale wars, like the Krim War and the two World Wars, caused great population turbulences and migrations and hence an intensive exchange of certain habits.
The financial scope of the citizens of many western countries in this century substantially increased and enabled them to spend more money on luxury goods like tobacco,

- a change of the cigarette smoke flavour from sharp and irritating to mild and aromatic, promoting inhalation of the smoke and hence an effective self-administration of tobacco's main pharmacological agent nicotine,
- aggressive and intensive advertisement campaigns, in which smoking is associated with all kinds of desirable situations and characteristics,
- the introduction of cigarette brands relatively poor in nicotine, necessitating the nicotine-dependent smoker to smoke more cigarettes to receive the amount of nicotine that he/she had got used to while smoking the older higher-delivery brands.

The total world production figure of raw tobacco amounted to almost 6 millions of tons in 1983.

The growing consciousness that inhalation of tobacco smoke is a prominent hazard to health is causing a shift in smokers' consumption patterns towards cigarettes low in tar and nicotine. It also tends to stabilize and even diminish tobacco sales figures in Western society.

Tobacco's 'active principle' was first isolated from leaves of *Nicotiana tabacum* by Posselt and Reiman in 1828. They isolated a volatile substance, pungent to taste and smell, which possessed the 'narcotic' properties of tobacco and was so toxic that 'a fourth drop was enough to kill a rabbit.' The substance was called nicotine in Jean Nicot's honour.

Nicotine has been used as an agricultural insecticide ('Black leaf 40') in the United States. In recent years, it has acquired its one and only therapeutic application as the main ingredient of a chewing gum that can be prescribed by physicians as a help in smoking cessation. The gum is most effective when used as part of a comprehensive program such as in smoking cessation clinics. Typical results from trials of this kind are 30-40% one-year abstinence compared to 15-20% for placebo-treated patients.

The use of nicotine gum in general medical practice is less encouraging. In one of two large studies having been reported, one-year abstinence was 9% for nicotine gum versus 4% for physician advice or no intervention. In the other, both approaches scored 10% one-year abstinence. Successful use of nicotine gum clearly depends on selection of highly motivated patients and on the motivation, experience, and time spent by physicians or other health care providers.
In pharmacokinetical terms, the failure of nicotine chewing gum to effectuate high cessation rates may very well be traced back to its failure to produce transient high plasma concentration peaks, as the gum releases nicotine only very gradually. If tobacco addiction indeed is a 'dependence on high nicotine bolus', a nicotine inhaler would have been a more adequate choice of formulation than a chewing gum.

A Note on Experiment-Induced Artifacts in Smoking Behaviour

One might think that, if by way of experiment, a volunteer were asked to smoke 'as he (or she) is accustomed to do', this would yield reliable data. Yet the very circumstances of the experiment often tend to modify the technique, in that the human guinea-pig is inclined to smoke more or less intensively than he usually does, with the result that the intake measurements will show an intake of toxic components, such as nicotine and carbon monoxide, that is not representative for the intake in everyday life of the experimental subject. Actually, this is comprehensible, for, if the persons undergoing the test were doing nothing else besides smoking, they would have kept their mind to it much more than in normal circumstances, this, together with their desire to co-operate and to meet the expectations of the experimentalist (if known to them), may easily have led to a more or less intense smoking technique.

In fact, there is no way to be absolutely sure that smoking pattern or intake of certain smoke constituents observed in the laboratory is representative for the everyday life smoking behaviour of the subjects. Nervousness in anticipation of the next venipuncture, boredom, the consciousness of being observed by the experimentalists, the supply of free cigarettes during the course of the experiment and many other factors which are very diverse in their nature induce changes in the smoking technique and pattern of experimental subjects. Even if the subject is allowed to smoke in his or her own environment and is merely requested to save the butts, one cannot be certain that even this noncomittal type of experiment will not affect smoking behaviour. The nasty smell that evolves from the container when adding a butt to the ones already saved, and the thought of their unsuspected high multiplicity might work out as a negative feedback mechanism that restrains the subject from lighting additional cigarettes too frequently and, when lighting one, makes one 'dawdle over' it.
However, if a subject's smoking behaviour is affected by so many circumstances in the laboratory, it is not unlikely to presume that many factors outside the laboratory affect it as well. Indeed, when collecting cigarette butts from filter cigarettes smoked by fully ambulant subjects over a 16 days period, large differences were found in the intra-individual daily average of the filter butt nicotine content. Obviously, there is no such quality as THE smoking behaviour of a cigarette smoker. Whether in experimental conditions or in everyday life, each cigarette is smoked individually, and the smoking parameters employed are a random indication.

Realizing this, there is no sense in eliminating every potential factor that could exert influence on a subject's smoking behaviour from the experiment. Factors existing in the 'outside world' should be maintained and imitated in the laboratory, and only clear-cut artificializing laboratory circumstances should be abandoned.

The gist of it is, as natural smoking is mostly done simultaneously with some other activity, in the laboratory the attention of the subjects should not constantly be focussed on the act of smoking, but rather be diverted from it by accompanying activities like reading, writing, drinking coffee or tea, playing cards, knitting, watching television or whatever. While doing so, they should not be observed, or at least not be aware of being observed, by the experimentalist(s). If available, a separate room is an ideal subject 'home' and an elegant solution for those scientists who wish to keep their laboratory free of nicotine contamination regarding possible future analyses of the volatile alkaloid. The volunteers should not be aware of the experimentalist's expectations or beliefs regarding the outcome of the experiments. They should not switch from one brand to another on experimentalist's request, and not be supplied any free cigarettes in the course of the experiment.

No matter its importance, in pharmacokinetical publications the psychosocio-economic design of smoking experiments is rarely discussed. In many instances these aspects of experimental design probably receive the attention they require, authors considering them too trivial or too detailed to mention in the methodology (which actually is the reason we discuss this matter in the preface of this thesis). In other instances authors do not seem to properly recognize possible artificializing conditions incorporated in their study design, nevertheless they often claim general applicability of their results and conclusions. We feel that the frequent omission of these details from publications has contributed to the diversity of the conclusions arrived at in studies with a seemingly similar design.
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CAFFEINE: NATURAL OCCURRENCE, ABUNDANCE, AND ITS HISTORY AS A DRUG OF INDULGENCE

"'t Swarte watervogt, dat alle kracht bezit,
Om 't sieckelijcke lijf gezondheid weer te geven.
Dat elcke kwael verdrijft, het koude bloed verhit,
En 't dichterlijk brein vervult met jeugdig leven."
('Tot lof van de koffij', Anonymus, 1792).

Caffeine, theobromine and theophylline are drugs which are similar in chemical constitution and pharmacological action. They are found in a large number of plants of several families and genera distributed widely throughout the
It is of interest to note that wherever these plants are indigenous, natives use their aqueous extracts as beverages. The consumption of some of these beverages such as coffee, tea, and cocoa is practiced all over the civilized world.

Coffee is obtained from the kernel of the dried ripe seed of Coffea arabica, C. liberica, C. canephora (robusta coffee), and other species of Coffea (Rubiaceae). The kernel is roasted until it acquires a deep brown color and a characteristic aroma. It contains about 1-2% of caffeine and small amounts of theobromine.

Tea, the prepared young leaves and leaf-buts of Camellia sinensis (= C. thea) (Theaceae), contains 1-4% of caffeine, some theobromine and traces of theophylline.

Cocoa is produced from the fermented and dry seeds of Theobroma cacao (Sterculiaceae) which contains in the kernel 1½-3% of theobromine and a small amount of caffeine. The shell contains about 1½% of theobromine. Heated and deprived of husk and membrane, the seeds yield cocoa nibs. The nibs with most of the solid fat (Theobroma oil) pressed out produce, when reduced to powder, cocoa for use as a beverage. The powder is often flavoured with vanillin or cinnamon.

In addition, a number of caffeine beverages, less universal in appeal, are consumed in widely scattered areas.

Mate is prepared from the dried leaves of Ilex paraguariensis (Aquifoliaceae) which contain 0.2-2% of caffeine. Mate is extensively used as a beverage in South America.

In the vast territory of the Sudan, natives chew the kola nut and swallow the extractives. The dried cotyledons of the nuts, from Cola nitida and C. acuminata (Sterculiaceae) contain 1½ - 2½% of caffeine and traces of theobromine. The kola nut is a valuable possession and is used as a basis for commercial exchange.

Many species of the Paullinia plant genus (fam. Sapindaceae) grow in the Amazon basin, but only two of these are used by the natives. Paullinia sorbilis as a beverage, and Paullinia cupana to prepare guarana, a dried paste prepared from the roasted seeds. The two species are unique in their genus in that they contain a large amount (more than 4% of the dry weight) of caffeine.

The earliest use of the xanthine beverages is lost in the obscurity of the past. However, legends telling of their discovery reveal the reason for their favor. For example, legend credits the discovery of coffee to a prior of an Arabian convent. Shepherds reported to the prior that goats which had eaten...
the berries of the coffee plant did not rest but gamboled and frisked about all through the night. The prior, mindful of the long nights of prayer which he had to endure, instructed the shepherds to pick the berries so that he might make a beverage from them. The success of his experiment is obvious from the popularity of coffee today, a billion pounds being consumed yearly in the United States alone.

Another story tells of the discovery of tea by Bodhidharma, who lived in the 6th century A.D. Bodhidharma, the son of a king of India and an apostle of Buddhism, traveled to China to spread his beliefs. There he lived under the open sky, mortified his flesh, tamed his passions, and subsisted on leaves. He sought sanctity by attempting to stay awake for 9 years while contemplating the virtues of Buddha. After three years of practice, he fell asleep. Upon awakening, overcome with chagrin, he cut off his eyelids so that his sin might not be repeated. A plant sprang up where his eyelids had fallen. He ate its leaves and found that he could return to his worship with renewed vigor. This plant was the tea plant.

From these old tales it can be inferred that caffeine is a stimulant. The name coffee is derived from the Arabic word 'gahwa' (pronounced as 'kahwah'), which was degenerated to kahweh by the Turks. This word means 'that which excites' or 'that which strives upward' and summarizes the pharmacodynamics of caffeine to a good extent.

It is beyond the scope of this thesis to unravel the history of all the above-mentioned xanthine-beverages. Therefore we will focus our attention on coffee, the most popular xanthine beverage in the western world and the most important source of caffeine in western diet.

It is highly probable that the province Kaffa in Abyssinia is the cradle of the coffee plant cultivation. During their military campaigns in the 13th and 14th century the Ethiopians brought it to Yemen, where it flourished well on the fertile terraces. Subsequently coffee travelled northward along the shores of the Red Sea to Mecca and Medina, from where the innumerable pilgrims spread its fame and its use to all directions. The boom of the Islam which was going on then and the strong expansion of the coffee-minded Turkish empire certainly stimulated the spreading of the coffee-drinking habit. In the 16th century coffee was a commonly used beverage in the whole Middle East. Women acquired the habit to an ever increasing extent. Turkish law even acknowledged the refusal of a husband to let his wife enjoy coffee as a solid reason to divorce.
In 1554 the first coffee-house on European soil was opened in Constantinopel, a century after its conquest by the Turks. Samples of coffee beans were subsequently introduced to Western Europe by enterprising merchants.

In 1645 the first Italian coffee-house was opened in Venice, in which city soon a comprehensive coffee culture arose. The introduction of the coffee into France passed off rather quietly. By 1676 Paris accommodated several coffee-houses, which were called 'cafés' after the name of the brew.

The coffee took Britain, presently the classic tea-country, by storm. The writer John Evelyn (1620-1706) mentioned a coffee-house in Oxford as early as 1643. About 1643 coffee had arrived at the status of being the favourite drink of the upper class and some thirty years later England counted more than 300 coffee-houses. One of them was Lloyd's Coffee-House, opened in 1688 by Edward Lloyd near the London sea-port, where over a cup of coffee many loading receipts and ship's insurances were discussed.

In the Low Countries, the interest for the coffee habit spread in the period from 1640 to 1670. Amsterdam soon became an important coffee trade center. In 1699 the at that time very young East-Indian Company established the first coffee estates in Java, where the Dutch cultivated tobacco and, later, Cinchona trees as well. These estates all were very successful, even today all around the world the name of Java is readily associated with high quality coffee and cigars. Coffee trade significantly contributed to prosperity in the Netherlands at that time, and coffee consumption became part of the luxurious life-style of the Golden Age. Probably the Dutch took the coffee to America while founding New Amsterdam (New York).

Today the annual world production rate of coffee beans is estimated to be 6 million tons. As coffee consumption enjoys unflagging popularity, rarely turns out to be addictive and is seldomly associated with adverse health effects by medical science, a further increase of mondial coffee consumption rate is anticipated.

The first publication reporting the isolation of caffeine from beans of *Coffee Arabica* by F Runge dates from 1820, and as early as 1875 L Medicus reported the clarification of caffeine's chemical structure. Classic pharmacological studies of (mainly) caffeine and related xanthines during the first half of this century have elucidated the stimulating and antisoporifor actions of this group of compounds, thereby confirming the ancient beliefs inferred by legend. Moreover, these studies have revealed that methylxanthines possess other important pharmacological properties as well. These properties were
exploited for a number of years in a variety of therapeutic applications, many of them have now been replaced by more effective agents. However, in recent years there has been an resurgence of interest in the therapeutic use of the natural methylxanthines and synthetical derivatives thereof, principally as a result of increased knowledge of their cellular basis of action and their pharmacokinetic properties.

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QUININE: NATURAL SOURCES, ABUNDANCE, AND THE HISTORY OF ITS APPLICATIONS

Quinine is the most abundant alkaloid occurring in cinchona bark, the bark of the roots, trunk, and branches of several species of the Cinchona genus of trees (Familia Rubiaceae) Best known are the species C. officinalis, C. succirubra, C. calisaya, and C. ledgeriana The alkaloid content of the dried bark of these species amounts 6-14 %, of which one third to one half is quinine Except quinine, more than 20 related alkaloids are found in cinchona barks, the most important of these being quinidine, cinchonine, and cinchonidine.
Cinchona trees grow wild on the eastern slopes of the Andes. The trees have special growth requirements, and their cultivation in other parts of the world has met with many difficulties. The early Jesuits ordered a new tree to be planted for each one stripped of its bark. Later, however, the indiscriminate exploitation caused the Cinchona trees to die, and the resulting high price of quinine during the middle of the nineteenth century was an impetus for planting the trees in other parts of the world. Both the British and Dutch governments spent considerable time and money studying the chemical and botanical aspects of the problem. The Dutch were more fortunate, and as a result the chief source (more than 90\%) of quinine today is from the trees cultivated in the former Dutch colony Java. The species *C. ledgeriana* is grown there, which yields the highest percentage of total alkaloids (10 - 14\%) and of quinine (5 - 8\%). Hybrid species, usually of *C. ledgeriana* and *C. succirubra*, bring forth a large proportion of the bark of commerce and yield a high percentage of quinine. Cinchona alkaloids presently are still obtained from these natural sources, since the laboratory synthesis of their complex chemical structure (including 4 chiral carbon centers) has appeared to be too elaborate and expensive a process to provide a practical source of these drugs.

It is not known whether the indigenous South-American population was acquainted with the pharmacological properties of cinchona bark. The first written record of the use of the bark occurs in a religious book written in 1633 and published in Spain in 1639. The author, an Augustinian monk named Calancha, of Lima, Peru, wrote "A tree grows which they call 'the fever tree' in the country of Loxa, whose bark, the color of cinnamon, is made into powder amounting to the weight of two silver coins and given as a beverage, cures the fevers and the tertians, it has produced miraculous results in Lima." A variety of colorful and fanciful versions of the discovery of the fever bark exists. A popular and persistent version is that the bark was employed in 1638 to treat Countess Anna del Chinchon, wife of the viceroy to Peru, and that her miraculous cure resulted in the introduction of cinchona into Spain in 1639 for the treatment of ague. There is no evidence that the countess ever used the bark, yet for many years the drug was called 'los Polvos de la Condesa.' However, the viceroy did bring a large shipment of cinchona bark to Spain. By 1640, the drug was being employed for fevers in Europe. Its use was first mentioned in European medical literature in 1643 by a Belgian physician, Herman van der Heyden.

The term cinchona was chosen by Linnaeus (who accidentally misspelled it) for the genus of plants yielding the drug. Although this term is probably
derived from the name of the countess whose alleged cure led to its wide use, some believe that it comes from a word of Incan origin, 'kinia', which means 'bark'. The Jesuit fathers were the main importers and distributors of cinchona bark in Europe, and the name 'Jesuit Bark' soon became attached to the drug. It was sponsored in Rome chiefly by the eminent philosopher Cardinal de Lugo, hence the drug there came to be called 'Cardinal's Bark'. The conservative medical groups viewed the new antipyretic with disdain because its use did not conform to the teachings of Galen. Others looked upon it with suspicion because the Jesuits used it. For these reasons, the drug was dispensed for many years predominantly by charlatans and in the form of secret remedies.

The first official recognition of cinchona bark came in 1677, when it was included in an edition of the 'London Pharmacopoeia' as 'Cortex Peruanus'. This early inclusion probably renders quinine the drug with the oldest pharmacopoeial history. For almost 2 centuries the bark was employed for medicine as a powder, extract, or infusion. In 1820, Pelletier and Caventou isolated quinine and cinchonine from cinchona bark, and the use of the alkaloids as such gained favor rapidly.

Until the third decade of the present century, the cinchona alkaloids represented the sole chemotherapeutic agents for the specific treatment of malaria. In the following years the use of quinine as an antimalarial was brought to a low ebb, a host of new synthetic antimalarials was developed starting from the chemical structure of quinine as a 'lead', especially when in the course of World War II quinine became scarce to the Allies. Many of them were more potent and less toxic than quinine, which exhibits myriads of more or less serious side-effects at the blood and tissue concentrations required for antimalarial activity. Indeed, quinine affects such a large variety of biological systems that it has been called a 'general protoplasmic poison', which appraisal, with a few reservations, is highly correct. It is toxic to many bacteria and other unicellular organisms such as trypanosomes, infusoria, yeast, spermatozoa and certain stages of development of the four Plasmodium species, which are the causative agents of malaria in man.

In the sixties, quinine, alone or in combination therapies, made its comeback as an antimalarial because of the prevalence of strains of Plasmodium Falciparum that had acquired multiple resistance or insensitivity to many or all synthetic antimalarial drugs in certain parts of the world. The lack of development of such resistance to quinine is ironic in view of the thousands of compounds that have been synthesized in an attempt to free the malaria patient from dependence on this relatively toxic natural product.
Other (rare) current applications of quinine are its employment as a reliever of recumbency leg muscle cramps (night cramps), and its occurrence as an additive of doubtful benefit in pick-me-ups and tonics.

In the sixties, the soft drink industry discovered quinine as a bitter principle that could be used for the flavoring of carbonated soft drinks and table waters like the well-known 'bitter lemon' and 'tonic' which may contain several dozens of milligrams of the drug per liter of beverage. In recent years the search for bitter agents that may substitute for the toxic alkaloid in soft drinks has begun. For the time being, its utilization in soft drinks labels quinine as a non-prescription, mass-consumed luxury drug. Consequently, quinine kinetics in healthy subjects, which significantly differs from those in malarious patients with clinical symptoms, should be investigated.

Literature cited

BRIEF SCOPE OF THE PRESENT DISSERTATION

Nicotine

• Investigation of the suitability of nicotine retention in smoked-out cigarette filters as a marker of mouth-level nicotine intake by smokers
• Investigation of the absolute intake of nicotine and carbon monoxide by habitual smokers of filter-tipped cigarettes
• Comparison of the yield of nicotine and carbon monoxide in human and in machine smoking of filter cigarettes
• Comparison of cigarettes with conventional and with ventilated filters with regard to their yield of the above compounds to smokers

Caffeine

• Investigation of caffeine pharmacokinetics after intravenous and oral administration to healthy volunteers
• Comparison of rate and extent of gastrointestinal caffeine absorption from coffee and from tea
• Investigation of paraxanthine pharmacokinetics after intravenous and oral administration in healthy volunteers
• Pharmacokinetical comparison of caffeine and its main metabolite paraxanthine
• Determination of the fractional conversion of caffeine into paraxanthine

Quinine

• Investigation of quinine pharmacokinetics after intravenous and oral administration to healthy caucasian volunteers
• Determination of quinine bioavailability in the most frequently administered oral formulation the sulphate salt in a gelatine capsule
GLOSSARY OF PROFESSIONAL TERMS, ABBREVIATIONS, AND SYMBOLS

* symbol of the convolution operator (see appendix II)

° indication of temperature: number of degrees centigrade

A(U)X Area Under the X(t) vs. time curve: $A(U)X = \int X(t) \, dt$

AUC Area Under the C(t) vs. time curve; zero statistical moment

AUC\textsubscript{or} AUC after oral administration

AUC\textsubscript{iv} AUC after intravenous administration

AUC\textsubscript{Z} AUC until the final (conc., time) data point at $t = Z$

BW body weight

C\textsuperscript{24} average circadian (24 hrs) plasma concentration

CAF caffeine

ch. chapter

cig. cigarette

CL total plasma clearance ($= \dot{V}_e$)

CL\textsubscript{m} metabolic plasma clearance ($= \dot{V}_{me}$)

CL\textsubscript{r} renal plasma clearance

CO carbon monoxide

CO\textsubscript{alv} CO concentration in expired alveolar air

%COHb percentage of hemoglobin monomers occupied by CO

fCOHb fraction of hemoglobin monomers occupied by CO

C.O. cardiac output ($= \dot{V}_B$)

conc. (plasma) concentration

conventional non-ventilated, non-perforated (filter cigarettes)

cot cotinine (subscript)

cotinine (-)-cotinine: main nicotine metabolite

cotinine-D2 4',4'-dideutero-(-)-cotinine

C(t) drug plasma concentration as a function of time

CV coefficient of variation; relative standard deviation

D input or dose; dose of nicotine reaching the oral cavity of the smoker

D\textsubscript{or}, D\textsubscript{iv} oral dose, intravenous dose

D(t), D(s) input rate or dosage rate function in time and Laplace domain, respectively

\dot{D} constant value of the input function D(t)

D\textsubscript{L,CO} amount of carbon monoxide absorbed into the pulmonary blood

DX (magnitude of) change of the value of quantity X

\Delta_{rel} relative deviation of $f_D$ and $f_D$,
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>extraction ratio, fraction of systemic drug eliminated during a single circulatory transit</td>
</tr>
<tr>
<td>$E_{\text{cot}}$</td>
<td>elimination rate of cotinine</td>
</tr>
<tr>
<td>ESTD</td>
<td>external standard</td>
</tr>
<tr>
<td>F</td>
<td>absolute nicotine retention in cigarette filter (cf D and $R_{\text{nic}}$), availability as estimated by plain AUC ratio (cf $h_a$)</td>
</tr>
<tr>
<td>$F'$</td>
<td>systemic availability as estimated by AUC ratio, corrected for individual changes in drug disposition rate</td>
</tr>
<tr>
<td>$F_1$, $F_2$, $F_3$, $F_4$</td>
<td>codes of the four conventional filter cigarette brands</td>
</tr>
<tr>
<td>$f_D$, $f_D'$</td>
<td>measured molar fractions of cotinine-D2 in a smokers' plasma sample and its duplicate, respectively</td>
</tr>
<tr>
<td>$f_L$</td>
<td>systemic availability of nicotine inhaled with tobacco smoke</td>
</tr>
<tr>
<td>$f_M$</td>
<td>fraction of a parent drug that is converted into a particular metabolite</td>
</tr>
<tr>
<td>$F(t)$</td>
<td>rest-of-body transport function, closed-loop feedback transport function (cf $H(t)$ and $\psi(t)$)</td>
</tr>
<tr>
<td>$F(s)$</td>
<td>rest-of-body transfer function, closed-loop feedback transfer function (cf $H(s)$ and $\psi(s)$)</td>
</tr>
<tr>
<td>$f_{\text{vol},X}$</td>
<td>fractional volume of gas X</td>
</tr>
<tr>
<td>g</td>
<td>gram, gravitational acceleration at sea level</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>$G(L)C$</td>
<td>gas (liquid) chromatograph(ic)</td>
</tr>
<tr>
<td>h, hr</td>
<td>hour</td>
</tr>
<tr>
<td>$h_a$</td>
<td>systemic availability as estimated by numerical deconvolution (cf $F$)</td>
</tr>
<tr>
<td>$Hb$</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>$H(t)$</td>
<td>heart-lungs transport function, closed-loop forward transport function (cf $F(t)$ and $\psi(t)$)</td>
</tr>
<tr>
<td>$H(s)$</td>
<td>heart-lungs transfer function, closed-loop forward transfer function (cf $F(s)$ and $\psi(s)$)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatograph(ic)</td>
</tr>
<tr>
<td>ID</td>
<td>internal diameter</td>
</tr>
<tr>
<td>ISTD</td>
<td>internal standard</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>$k$</td>
<td>power of time in a pharmacokinetic gamma function</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>MAT</td>
<td>mean absorption time ($= TH_a$)</td>
</tr>
<tr>
<td>MDT</td>
<td>mean dosage time ($= TD$)</td>
</tr>
</tbody>
</table>
MedAT  median absorption time
MET  mean (urinary) excretion time
min  minute
MIT  (in general) mean input time (MIT = MDT + MAT in case of oral
dosage, MIT = ½ * infusion time in case of i v infusion, MIT =
0 in case of pulse i v injection)
ModAT  modal absorption time
MRT  Mean (body) Residence Time
NS-SIM  mass spectrometry - selective ion monitoring
MTT  mean (body) transit time
nic  nicotine (subscript)
NDIR  non-dispersive infra red
NSD  nitrogen-sensitive detector or - detection
N<sub>rc</sub>  average number of body circulations
p, PP  page, pages
P  type I error probability (of rejecting the null hypothesis
while it is true)
op  per os, peroral
ppm  parts per million 1 ppm = a partial volume of 10<sup>-6</sup>
PX  paraxanthine (main caffeine metabolite)
Ψ(t)  total body transport function, probability density distribution
of body residence times
Ψ(s)  total body transfer function, the Laplace transform of Ψ(t)
Q  quinine
Q<sub>r</sub>(t)  amount of quinine renally excreted at time = t
r  coefficient of correlation
R  analytical recovery of a drug
R<sub>nic</sub>  relative retention of nicotine in the cigarette filter
R<sub>nic</sub> = F/(F + D)
®  registered trade mark
s  second, or transform variable of time in the Laplace domain
SD  standard deviation
SIM  selective ion monitoring
SOC  steroid oral contraceptives
SPE  solid phase extraction
t  time
t<sub>½</sub>  half-life of plasma elimination of a drug

xxxvi
T'AUC area under the time-concentration vs. time curve: 1st statistical moment
T^2AUC area under the squared-time-concentration vs. time curve: 2nd statistical moment
TB theobromine (minor caffeine metabolite, also occurring in chocolate, tea and coffee)
TP theophylline (minor caffeine metabolite, also occurring in minute amounts in tea)
TX mean transit time of the function X(t): TX = \int t \cdot X(t) dt / AX
\tau exponential time constant occurring in the equation C(t) = A \cdot e^{-t/\tau}
\theta times of urine voiding
u time of last oral data point included in numerical deconvolution calculations of h', MAT, MedAT, and ModAT
UFR(x) urine flow rate (in the first x hours following administration)
UV ultra violet
V volume
\dot{V} volume differentiated with respect to time: flow
V1, V2 codes of the two ventilated filter cigarette brands studied in this thesis
V_B total blood volume (= 0.08 l/kg body weight in man)
\dot{V}_B total blood flow; cardiac output (= C.O.)
V_dss apparent volume of distribution
\dot{V}_{el} total plasma clearance (= CL)
\dot{V}_{mel} metabolic plasma clearance (= CL_m)
\dot{V}_{hep} hepatic blood flow
VR ventilation rate (% of vent air in the mainstream of the smoke)
VR_{nom}, VR_{min} nominal (maximum) and minimal ventilation rate of a ventilated filter cigarette, respectively
VRT Variance of (body) Residence Times
vs. versus
VX variance of transit times of the function X(t):
VX = (\int t^2 \cdot X(t) dt) / AX - (TX/AX)^2
WSS summation of weighted squared deviations of concentrations
PHARMACOKINETICS: A SYSTEMS DYNAMICS APPROACH
1.1 INTRODUCTION

The human body may be considered as a dynamical system. It is not only reacting to inputs from the outside world, but it is also a self-organizing system, to some extent controlling its input and even controlling its environment.¹

Obviously, a large number of linear and nonlinear feedback control systems regulating posture, movements, blood pressure, etc. is involved. To understand such a complicated system, it is logical to apply knowledge gained in systems dynamics and control theory. Recent developments in the applied mathematics of dynamical systems may greatly facilitate the study of the behavior of the body and its responses to the input of drugs.²,³

1.2 DYNAMICAL SYSTEMS THEORY OF BODY FUNCTIONING

In systems dynamics, the body can be characterized by a large number of state variables: the state vector $X$.⁴ Depending on the type of study one does, one may consider various state variables since it is impossible to consider all state variables at the same time. State variables may be the blood pressure in the aorta, the core temperature, the autonomic drive to the intestine, the tension in the sensors of a muscle, the degree of muscle contraction, the hypothalamic drive on the growth hormone of the hypophysis, etc. See figure 1.

The future state of the system depends on the present state, the memory of the system, the input and the time. The change in the state variables is therefore a function of the state $X$, the time $T$, the memory $M$, and the input $I$:

$$ \dot{X} = F(X, T, M, T) $$
STATE VARIABLES: $X$
- Blood pressure $X_1$
- Core temp $X_2$
- Insulin conc $X_3$

FIGURE 1 Schematic presentation of the body as a dynamical system. Many thousands of state variables together may fully characterize the entire body behavior. Such a multidimensional dynamical system would be impossible to study were it not that attractors are involved by which the actual behavior is of a much lower dimension.

This relationship may be highly nonlinear so that the differential equation cannot be solved by classic methods, but new ways of analysis have been developed.

Although the system is of an extremely high dimension, it behaves as a relatively low-dimensional control system. This implies that attractors are involved. In the course of time, the systems settle down to point attractors, periodic attractors (oscillation), or so-called strange attractors.

1.3 THE BEHAVIOR OF PHYSIOLOGICAL SYSTEMS IS DOMINATED BY ATTRACTORS

The multidimensional state space of a living system is dominated by asymptotic behavior of much lower dimension. Attractors may be considered as low-dimensional subsets of the state space to which its behavior quickly settles down.

In this way, the dynamical system can be visualized by its phase portrait, that is, the state space of the systems can be divided in basins, which are the areas of influence of its attractors separated by lines of bifurcation or separatrices.
The best known attractor is the point attractor. It is the steady state of a system. Garfinkel\(^3\) recently pointed out that thinking in physiology has been dominated by point attractors; so students in medical schools are still taught the importance of homeostasis, whereas reality is much more complicated.

Periodic attractors are the basis of oscillation, which is abundant in biology (ECG, EEG, etc.). The cyclic behavior of dynamical systems on basis of periodic attractors is reasonably well understood.

The interest now is on aperiodic, chaotic, or strange attractors. A nice clarification has been given by Hofstadter.\(^6\) Details can be found in an article by Abraham and Shaw\(^5\) and by Ott.\(^7\)

1.4 DYNAMICAL SYSTEMS THEORY IN PHARMACOKINETICS

In pharmacokinetics one may often disregard a large number of the state variables of the system, since they are irrelevant for the understanding of the disposition of drugs in the body. The relevant state variables in pharmacokinetics are the concentration of drugs in the aorta, the central venous pool, the liver sinusoids, etc. The differential equation may even be a linear one:\(^8\)

\[
\dot{X}(t) = A*X(t) + B*I(t)
\]

where \(X(t)\) is the state vector, \(I(t)\) the input vector, and \(A\) and \(B\) matrices. If there is linearity, the differential equation can be solved:\(^8\)

\[
\dot{X}(t) = \phi(t) * X(0) + \phi(t) * B*I(t)
\]

The further state behavior is completely determined by the state transition matrix \(\phi(t)\), the initial state and the input. Also the output of the system is uniquely described. The attractor of the system is a point attractor so that a constant input always leads to a steady state situation.

For a better understanding of the systems dynamics approach in pharmacokinetics and the implications of various input functions, we will first consider the subsystems of the body, which are the individual organs and tissues.
In contrast to the total body system, the subsystems are relatively homogenous, therefore, only a few state variables fully characterize those systems.

15.1 A Drug Transport in Tissues and Organs

When a drug is injected into the blood supply of a tissue or organ, the individual drug molecules may pass the tissue via quite different routes. The transit time, that is, the time from entry to exit for the individual molecules depends on the length of the pathway and whether or not the molecules enter cells surrounding the blood vessels and remain there for some time. Some molecules may leave the tissue already after a few seconds whereas others may have a much longer transit time.

Figure 2 is an illustration of a two-dimensional network of a part of the wing of a bat. It may be seen from this two-dimensional network that even drug molecules that do not leave the lumen of the blood vessels may have quite different transit times as some molecules may even temporarily proceed in the wrong direction. Obviously, a much larger variation may occur in all mammalian organs and tissues as the capillary network is mostly three-dimensional and the bloodflow is not the same all the time.

15.2 B The Density Function of Transit Times

With regard to drug transport, a tissue or organ may be characterized by a density function of transit times. This will be a continuous distribution function, since even the slightest amount of drug (say 1 µg) consists of a huge number of molecules (more than $10^{18}$). For each subsystem, therefore, a density function of transit times is the characteristic transport function which dictates what occurs on the venous site when drug is offered to the arterial site. See figure 4.

Figure 3 is an illustration of the output concentration of bromfluorescein when given by a pulse injection to a lung lobe of a dog. The input pulse is dispersed by the subsystem analogous to the dispersion in a liquid chromatograph. In general, there is a delay or lag-time, a steep rise, a peak, and a decline. See figures 3 and 4.
FIGURE 2. Two dimensional capillary network of part of the wing of a bat. Molecules entering at the arterial site may pass the network via different pathways. In addition, they may leave the capillary and remain in the cells surrounding the capillaries. Consequently the transit time of the molecules will be distributed according to a certain density function. A realistic density function for this system is shown in figure 4. (From Nicoll, P.A. and Webb, R.L., Annals N.Y. Acad. Sci. 46 (1946) 697. With permission).

Obviously, when a drug is given as a very small pulse, all drug molecules enter the system at the same time and the output curve is directly proportional to the density function of transit times. The transport function of a subsystem may therefore be regarded as the unit impulse response of that subsystem.

As an abstraction of its shape, the density function of transit times of a subsystem can be characterized by the modal transit time (ModTT; the time of peak occurrence), the median transit time (MedTT, the time at which half of the molecules have passed), and the mean transit time (MTT, the sum of all individual transit times divided by the number of molecules leaving the subsystem). See figure 4. In addition, the volume of the subsystem (V), the bloodflow though it (\dot{V}) and, in case of elimination, the extraction ratio (E) are of importance.
FIGURE 3 Arterial concentration input function and the venous concentration output function of a fluorescent dye in a part of the lung. The mean transit time is ±3 sec (Reproduced from Wagner, W.W. et al., Science 218 (1982) 379 With permission).

FIGURE 4 Density function of transit times $F(t)$ and its integral, the probability function $P(t)$. In this case the modal transit time is 7 sec, the median transit time is 10 sec, and the mean transit time is 12 sec. Some molecules may have very large transit times, which have a considerable influence on the mean transit time.
In a subsystem, the central volume principle requires that the volume equals the product of the flow and the mean transit time. This principle is generally applied in circulation research.

1.5.3 Flow Vessel Analog of a Tissue

A flow vessel is a reasonably good analog of a tissue or organ. See figure 5 (left). If the contents of the vessel are thoroughly mixed, the drug concentration is the same in all parts of the vessel. As a result, the decrease in the concentration following a pulse input at the entrance is proportional to the concentration in the vessel. This implies that the transfer function is exponential. See figure 5 (right).

\[
F(t) = \frac{1}{\tau} e^{-\frac{t-T}{\tau}} \quad \text{and} \quad F(s) = e^{-Ts} / (st + 1)
\]

where \( \tau \) is the time constant and \( T \) is the delay. The mean transit time of the flow vessel equals the time constant. It is interesting to note that the median transit time equals the half-life, while the modal transit time equals the delay.

In the actual in vivo situation, the rise will be less steep and the decline not exactly exponential. The flow vessel is, however, a good model of a tissue or organ.

1.6 CONTROL SYSTEMS DYNAMICS OF A SUBSYSTEM

A subsystem is fully described by its transport function \( F(t) \), which is in fact a density function of transit times. Control systems theory can be used to understand the input-output relations. See figures 2, 5 and Table 1. According to conventions in systems dynamics, the input and the output can be considered in the time domain as well in the Laplace s-domain. The transport function \( F(t) \) in the time domain corresponds to the transfer function \( F(s) \) in the Laplace s-domain. See Table 1.

The relationship between the input function \( D(t) \), which is a dosage flow of drug (e.g., in \( \mu g/min \)), and the output function \( C(t) \), which is the concen-
FIGURE 5. (Top left) Block diagram of a subsystem with simple input and simple output. (Bottom left) A good example is a flow vessel. The volume and the flow, provided that mixing is efficient, fully determine the subsystem behavior. (Right) Density function of transit times of an ideal flow vessel. This function is monoexponential, so its median transit time equals the half-life time while the mean transit time is \( \frac{1}{\ln 2} = 1.44 \) times the half-life. In this example the MTT = TF = 2 s.

Concentration in the venous blood leaving the subsystem (e.g., in \( \mu \text{g/ml} \)), is governed by the transport function \( F(t) \) and the bloodflow (\( \dot{V} \)):

\[
C(t) = \left( \frac{1}{\dot{V}} \right) D(t) * F(t) \quad \text{and} \quad F(s) = \left( \frac{1}{\dot{V}} \right) D(s) * F(s)
\]

where \( * \) is the symbol of the convolution integral. See Table I.

If elimination occurs in the subsystem, e.g. as in the liver and the kidneys, the extraction ratio is also involved. See Table I. In general, the output is the convolution of input function and the transport function in the time domain, and merely the product of input function and transfer function in the Laplace domain.

The transport function (that is, the density function of transit times) may resemble a log normal distribution, a gamma function, a Poisson distribution, or a sum of exponentials. The analysis is obviously best if one needs not to take into account what kind of function is involved. This implies that integral analysis, using the entire curve, is of advantage.
Table I
SYSTEMS DYNAMICS IN A SUBSYSTEM

The systems equation

**Laplace domain**

\[ C(s) = \frac{D(s)}{V_B} \cdot F(s) \quad \text{Without extraction} \]

\[ C(s) = \frac{D(s)}{V_B} \cdot (1-E) \cdot F(s) \quad \text{With extraction} \]

**Time domain**

\[ C(t) = \frac{D(t)}{V_B} \cdot (1-E) \quad F(t) = \frac{(1-E)}{V_B} \int_0^t D(t-\lambda) \cdot F(\lambda) \cdot d\lambda \]

where * is the operator symbol of the convolution integral

**Analysis**

**Areas**

\[ AC = \frac{AD}{V_B} \cdot AF \quad AC = \frac{AD}{V_B} \cdot (1-E) \cdot AF \]

\[ AUC = \text{dose} \quad AUC = \frac{\text{dose}}{V_B} \cdot (1-E) \]

**Mean times**

\[ TC = TD + TF \quad \text{where } TF = MTT \]

**Variance**

\[ VC = VD + VF \]

**Volume of distribution**

\[ V = \dot{V}_B \cdot MTT \]

Note: \( D(t) \) is the dosage input function (e.g., in mg/h), \( F(t) \) is the subsystem transport function which is the density function of transit times, \( \dot{V}_B \) is the bloodflow, \( E \) the extraction ratio. For the explanation of the other symbols, see Table II. The symbol "A" in \( AC, AD, \) and \( AF \) stands for Area under the \( C(t), D(t) \) and \( F(t) \) curve. In analogy, the "T" and the "V" stand for the mean Time and its Variance, respectively, of the functions \( C(t), D(t) \) and \( F(t) \).

A input dosage flow into the subsystem. Output dosage flow out of the subsystem. Unit of both input and output e.g., mg/h. The output equals the blood flow through the subsystem (l/h) times the concentration in the blood leaving the subsystem (mg/l).

16.1 A Analysis of Input-Output Relations

In systems analysis, we use the statistical moments as a useful type of integral analysis, disregarding the shape of the functions involved.
Table II

ANALYSIS OF TRANSPORT AND TRANSFER FUNCTIONS BY STATISTICAL MOMENTS

Function, in the time domain $F(t)$, in the Laplace domain $F(s)$

Definition of the $k^{th}$ general moment:

$$m_k = \int_0^\infty t^k \cdot F(t) \cdot dt$$

For a density function of transit times:

$m_0 = 1$ (the area) $m_1 = TF = MTT$ (mean transit time) or the expected value $E(F(t))$

Definition of the $k^{th}$ central moment:

$$\mu_k = \int_0^\infty (t-m_1)^k \cdot C(t) \cdot dt$$

For a density function of transit times:

$$\mu_0 = 1 \quad \mu_1 = 0 \quad \mu_2 = VF = the \ variance$$

Symbols used in the text:

Areas

$$AF = \lim_{s \to 0} F(s) = \int_0^\infty F(t) \cdot dt$$

Mean time

$$TF = - \lim_{s \to 0} \frac{d \cdot \ln F(s)}{ds} = \int_0^\infty t \cdot F(t) \cdot dt/AF$$

Variance

$$VF = \lim_{s \to 0} \frac{d^2 \cdot \ln F(s)}{ds^2} = \int_0^\infty t^2 \cdot F(t) \cdot dt/AF - TF^2$$

See Table I. We then obtain directly the area under the curve of the functions, the mean times, and their variance. There is a simple relationship between the moments and the so-called final value theorem of the Laplace transforms.\(^{13,15}\) See Tables I and II.

Whatever the input function $D(t)$ may be, its area equals the total dose administered. The mean time of the input function is the mean time for the dose to enter the subsystem. Obviously, the mean input time is zero if the dose is given as a short lasting pulse.
FIGURE 6. The influence of various types of input functions on the output of a subsystem. (Top) The input is a pulse or Dirac Delta function. This implies that all the molecules enter at the same time so that the output is directly proportional to the transport function so convolution reduces to multiplication. (Middle) The input is a step function or constant infusion. The output is the integral of the input function, so the convolution integral reduces to a simple integration. (Bottom) Any input function. As a result the output is the convolution integral of the transport function and the input function.

The area under the transport function F(t) is obviously equal to unity as is the case in a density function, because the integral is the probability function. The mean time of the transport function is the mean transit time of the subsystem.

The variance of the functions D(t) and F(t) is the variance of the mean input time and the mean transit time, respectively.
### Table III

**SPECIAL INPUT FUNCTIONS INTO A SUBSYSTEM**

**General equation**

\[ C(s) = \frac{D(s)}{V_B} \cdot (1-E) \cdot F(s) \quad \text{and} \quad C(t) = \frac{D(t)}{V_B} \cdot (1-E) \cdot F(t) \]

1) **Pulse input** \((D(t) = D\delta(t))\)

\[ C(s) = \frac{D}{V_B} \cdot (1-E) \cdot F(s) \quad C(t) = \frac{D}{V_B} \cdot (1-E) \cdot F(t) \]

2) **Step function input** \((D(t) = D)\)

\[ C(s) = \frac{D}{s} \cdot (1-E) \cdot F(s) \quad C(t) = \frac{\dot{D}}{V_B} \cdot (1-E) \int_0^t F(\lambda) \, d\lambda \]

**Steady state situation** \((t=\infty)\)

\[ C(\infty) = \frac{\dot{D}}{V_B} \cdot (1-E) \]

**Note:** \(\delta(t)\) = the Dirac delta unit impulse function, \(\dot{D}\) = the constant step function input rate, \(C(\infty)\) = the concentration at steady state

---

16.2 B Special Input Functions to a Subsystem

The Dirac \(\delta\)-pulse. A pulse injection is a special input, since in that case all the molecules enter the subsystem at the same moment. The output then is directly proportional to the transport function. This implies that the convolution operator then reduces to a proportionality. See figure 6 and Table III. The proportionality factor is the area under the output (concentration) curve. So, the transport function \(F(t)\) can be calculated simply by dividing the output concentration by its area under the curve following a pulse injection.

The step function input. A constant infusion or step function is also a special input. It may be regarded as a sequence of pulse injections of equal size applied at regular time intervals. The convolution operator then reduces to a normal integration. See figure 6 and Table III. If the constant infusion is continued over a sufficient long period of time, that is, long with respect to the mean transit time, the output concentration is also constant and equal to the quotient of the constant dosage flow (that is, the input rate) and the bloodflow. See Table III. Obviously, if extraction occurs, the output would be lowered by a factor equal to \((1-E)\) since only that fraction passes the subsystem intact. Since the central volume principle holds,
the amount of drug in the tissue during steady state is also constant and is equal to the product of the dosage flow and the mean transit time. See Table III.

Any input system. The input system in general can be dissected in a number of injections of different size given in succession. The output of each injection is proportional to the dose and has the shape of the transport function. The overall result is the superposition of all these curves, which in fact is the convolution integral.

163 C The Output in the Steady State

Depending on the dynamics of the transport function, the steady state will be reached rapidly or more slowly. The dynamics of small, highly vascularized tissues are fast as reflected by a small MTT of seconds or so that the output of such a tissue practically follows the input and steady state is reached very rapidly. The dynamics of poorly vascularized tissues are much slower, in the order of several minutes, and their output will not readily follow input variations.

The dynamics of individual tissues may vary greatly, but their dynamics are, in general, much faster than the kinetics of drugs in the entire system. The mean transit time of drugs in tissues and organs is from several seconds to several minutes, whereas the mean residence time of drugs in the body is in the order of a few hours to many hours. Obviously, for the total system it is of great importance how the various subsystems as the organs and tissues are arranged. See handbooks of anatomy, e.g., Gray's 49.

17 THE ARRANGEMENT OF SUBSYSTEMS

In principle, the subsystems may be arranged in series or in parallel. The output of such an arrangement may be fed back as input to the system (closed-loop system), or it may not be fed back (open-loop system). For reasons of simplicity, we will restrict ourselves here to two subsystems with transport functions $F_1(t)$ and $F_2(t)$. For more complicated arrangements, see handbooks such as Di Stefano et al. 12.
FIGURE 7 Arrangement of subsystems in the time (t) domain (Top) Two subsystems in series. In the Laplace or s domain, the overall transfer function is the product of the two transfer functions. (Middle) Two subsystems in parallel. In the Laplace domain, the overall transfer function is the weighted sum of the two transfer functions. The weighing factor is the blood flow through each of the subsystems. (Bottom) A subsystem with positive feedback arrangement. The output is fed back to the input site. The overall transfer function is typical for a positive feedback control system.

17.1 A Subsystems in Series

If two subsystems are connected in series, the output of the first is input to the second. See figure 7 (Top). Consequently, the overall transport function is the convolution of the two transfer functions of the subsystems

\[ F(t) = F_1(t) * F_2(t) \]

The flow to the two subsystems is equal, so that the overall system equation can easily be written down. See Table 11. If a pulse injection is given to the first system, there is dispersion in the first, while the second system causes...
a further dispersion. See figure 8. Obviously, the mean transit time of the total system equals the sum of the mean transit times of the two subsystems, and the delays in the subsystems add to a total delay.

FIGURE 8. (Top left) Individual monoexponential transfer functions $F_1(t)$ and $F_2(t)$ with time constants of 1 and 4 min, resp. (Top middle) The same functions on a log scale. (Bottom left) The result of the arrangement of $F_1(t)$ and $F_2(t)$ in series (cf. figure 7, upper panel) and in parallel ($f_1 = 0.8$, $f_2 = 0.2$; cf. figure 7, middle panel). Note the great difference in the two curves. (Bottom middle) The same functions on a log scale showing that the two time constants of 1 and 4 min still dominate the overall curves. (Top right) The forward path and the first five recirculations of the feedback arrangement (cf. figure 7, lower panel; $\tau$ here equals 10 s and $\Theta$ is 4 s). The curves grow flatter and the delays are added. (Bottom right) The overall output is the superposition of all the individual curves. Note that after initial slight oscillations the curve attracts to a fixed point simply because recirculation mixing occurs very rapidly.
Table IV
ARRANGEMENT OF SUBSYSTEMS

General systems equations
\[ C(s) = \frac{D(s)}{V} \cdot F(s) \quad C(t) = \frac{D(t)}{V} \cdot F(t) \]

1) Two subsystems in series
\[ F(s) = F_1(s) \cdot F_2(s) \quad F(t) = F_1(t) \cdot F_2(t) \]

Analysis
Areas
\[ AC = \frac{AD}{V} \cdot AF_1 \quad AF_2 = \frac{dose}{V} \]

Mean times
\[ TC = TD + TF_1 + TF_2 \quad MTT = TF_1 + TF_2 \]

2) Two subsystems in parallel
\[ F(s) = f_1F_1(s) + f_2F_2(s) \quad F(t) = f_1F_1(t) + f_2F_2(t) \]
\[ (f_1 + f_2) = 1 \]

Analysis
Areas
\[ AC = \frac{AD}{V} (f_1AF_1 + f_2AF_2) = \frac{dose}{V} \]

Mean times
\[ TC = TD + \frac{f_1TF_1 AF_1 + f_2TF_2 AF_2}{f_1AF_1 + f_2AF_2} = TD + f_1TF_1 + f_2TF_2 \]

Note: \( A \) and \( T \) denote the area and the mean time of the function \( X(t) \), respectively. \( X(t) = C(t), D(t), F_1(t) \) or \( F_2(t) \)

17.2 B Subsystems in Parallel

The input is divided over the two subsystems simultaneously dependent on their relative flows. The total transfer function is the weighted sum of the transfer functions of the subsystems
\[ F(t) = f_1F_1(t) + f_2F_2(t) \]
Table IV (continued)

ARRANGEMENT OF SUBSYSTEMS

3) A subsystem in positive feedback arrangement

<table>
<thead>
<tr>
<th>Laplace domain</th>
<th>Time domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>First pass</td>
<td></td>
</tr>
<tr>
<td>( C_1(s) = \frac{D(s)}{V_B} \cdot F(s) )</td>
<td>( C_1(t) = \frac{D(t)}{V_B} \cdot F(t) )</td>
</tr>
<tr>
<td>Second pass:</td>
<td></td>
</tr>
<tr>
<td>( C_2(s) = \frac{D(s)}{V_B} \cdot F(s) \cdot F(s) )</td>
<td>( C_2(t) = \frac{D(t)}{V_B} \cdot F(t) \cdot F(t) )</td>
</tr>
<tr>
<td>( C_n(s) = \frac{D(s)}{V_B} \cdot F(s)^n )</td>
<td>( C_n(t) = \frac{D(t)}{V_B} \cdot F(t)^n )</td>
</tr>
</tbody>
</table>

Summation of all passes:

\[ C(s) = \frac{D(s)}{V_B} \cdot \frac{F(s)}{1 - F(s)} \]
\[ C(t) = C_1(t) + C_2(t) + \ldots \]

Attractor following a single dose \( D \):

\[ C(\infty) = \frac{D}{V_B} \cdot \frac{1}{MTT} \]

Note: 1) In case no elimination occurs, a steady-state level is reached following a single dose, so the attractor is a point attractor. 2) The equation after one pass equals the open-loop or general systems equation.

where \( f_1 \) and \( f_2 \) are the fractions of the flow for the two subsystems. The overall systems equation is given in Table III. The mean transit time is also the weighted sum of the transit times of the two subsystems.

An example is given in figure 8. For instance, if both \( F_1(t) \) and \( F_2(t) \) are mono-exponential transfer functions, the overall transfer function is biexponential.

1.7.3 C. Subsystems in Feedback Arrangement

If the output of a subsystem is fed back to the system again, the next output is again the result of a convolution operation. Repetitive passages through the subsystem causes progressive dispersion. See figure 9. In this figure, a pulse is given to the system showing an exponential dispersion during the first pass. It is like feeding back the output of a liquid chromato-
The progressive dispersion results in a constant concentration after many recirculations. See bottom of figure 9. This is the attractor of the recirculation system following a single input. See Table IV (continued).

It is obvious that if, during a single pass, some degree of extraction occurs, the point attractor will be zero as finally the drug has left the system.

18 SYSTEMS DYNAMICS OF THE TOTAL BODY

The behavior of the total body system depends on the behavior of the subsystems and anatomical organization of the subsystems. See figure 9.

18.1 A Anatomical Organization of the Subsystems

The various organs and tissues of the body that receive arterial input are in essence arranged in parallel. The liver is an exception, since it is in series with the small intestines. See figure 10. According to control systems theory, the overall transfer function of a system consisting of subsystems arranged in parallel is an addition of the individual subsystem transfer functions. The transfer function of subsystems in series are to be multiplied.

We have grouped together all tissues and organs that receive arterial input, while the gut and the liver in series is considered as one subsystem. See figure 11. The heart-lung system is considered as a separate subsystem that is arranged in series with the rest of the body. See figure 11. If one injects a drug into the vena cava, molecules first pass the heart-lung system. So, if one observes the concentration in the aorta, one sees the dispersion or distortion brought about in the heart-lung system. The drug molecules then pass on to the rest of the body, again being dispersed. Still observing in the aorta, one sees after some time again the dispersed concentration or distortion after the second pass through the heart-lungs. In essence, one sees the result of several recirculations. The concentration, however, finally dies out as during each pass through the body a certain fraction of drug is eliminated. This fraction is called the extraction ratio. In general, extraction occurs not in the heart-lung system, but in the rest of the body in which the liver...
FIGURE 9. Semischematic anatomical arrangement of organs and tissues with their blood supply. The system is 'viewed' by measuring the concentration of a drug in the blood of the aorta or some peripheral venous observation sites.

and the kidneys are included. The observed concentration of drug in the aorta, when a drug input function is given to the vena cava, is therefore the sum of a series of convolutions because of the recirculations. The sum of the products reduces to a simple equation showing a positive feedback system. In fact, conventions of control systems theory immediately let one write down the total systems equations of the systems of figure 12. See Table V.
FIGURE 10. Diagram of the arrangement of the most important tissues and organs. Most tissues receive arterial blood and are therefore arranged in parallel. The blood leaving these tissues is pooled in the vena cava from which it flows to the heart and the lungs. The lungs are therefore in series with the tissues of the rest of the body. An overall system equation can easily be derived. (From Van Rossum, J.M. et al., Pharmacol. Ther. 21 (1983) 77. With permission).

FIGURE 11. Reduced block diagram of the body. All the tissues that are arranged in parallel have been grouped together. In essence this results in a simple positive feedback arrangement with the lungs (transport function $H(t)$) in the forward path and the organs and tissues of the rest of the body (group transport function $F(t)$) in the feedback path.
THE POSITIVE FEEDBACK BODY TRANSFER FUNCTION

Systems equation

Laplace domain

\[ C(s) = \frac{D(s)}{V_B} \cdot \frac{H(s)}{1 - (1-E) \cdot F(s) \cdot H(s)} = \frac{D(s)}{V_{el}} \cdot \psi(s) \]

where:

\[ \psi(s) = \frac{E \cdot H(s)}{1 - (1-E) \cdot F(s) \cdot H(s)} \]

Time domain

\[ C(t) = \frac{D(t)}{V_{el}} \cdot \psi(t) \]

Analysis

Areas

\[ A_C = \frac{AD}{V_B} \cdot \frac{AH}{1 - (1-E) \cdot AF \cdot AH} = \frac{\text{dose}}{V_B \cdot E} = \frac{\text{dose}}{V_{el}} \]

Mean times

\[ T_C = T_D + T\psi = T_D + \text{MRT} \]

where:

\[ \text{MRT} = \text{TH} + \text{MTT} \cdot \frac{1 - E}{E} = \frac{\text{MTT}}{E} - \text{TF} \]

and:

\[ \text{MTT} = \text{TF} + \text{TH} \]

Note: \( \psi(s) \) = the normalized overall positive feedback transfer function. \( \psi(t) \) = the total body transport function, i.e., density function of residence times. \( \text{MTT} \) = the body mean transit time, i.e., the mean time for a single pass. \( \text{MRT} \) = the body mean residence time. \( V_B \) = the cardiac output. \( E \) = the extraction ratio. \( V_{el} \) = the clearance.

Input: dosage flow directly into the mixed venous pool. Output: drug flow through the aorta = conc. in the aorta times \( V_B \).

It may be emphasized here that the study of pharmacokinetics essentially deals with the result of the recirculations. Circulation research mainly aims at the study of the density functions of transit times of individual organs and tissues or the total system. The circulation times of the tissues and organs is of the order of seconds to minutes, whereas the time con-
stants of the residence of drugs in the body are in the order of hours to
days In accordance, data sampling in pharmacokinetics is not frequent, in
contrast to data sampling in circulation research

Table VI
THE TOTAL BODY POSITIVE FEEDBACK CONTROL SYSTEM OF DRUG
DISPOSITION

\[ H(s) \] The forward path transfer function
\[ F(s) \] The feedback path transfer function
\[ F(s)\cdot H(s) \] The open-loop or single pass transfer function
\[ F(t)\cdot H(t) \] The transport function governing a single transit through
the body It therefore is a density function of body
transit times
\[ \ast \] Symbol for the convolution integral
\[ H(s) \] The closed-loop transfer function governing the
repetitive transit through the body
\[ 1-(1-E)\cdot F(s)\cdot H(s) \] The steady-state error of the closed-loop feedback system
\[ 1/E \] The normalized closed-loop transfer function (normalized
by multiplication with \( E \))
\[ \psi(s) \] The transport function governing repetitive transport
through the body It therefore is a density function of
body residence times
\[ E \] The extraction ratio, i.e., the fraction of drug
eliminated during a single pass
\[ V_B \] The cardiac output

182 B The Feedback Control System

The output of the aorta is fed to the rest of the body with transfer
function \( F(t) \), while its output is again is again fed back to the mixed venous
pool The result is a positive feedback with a forward transfer function \( H(t) \)
and a feedback transfer function \( F(t) \) The two in succession give the open-
loop transfer function governing a single pass through the entire body See
Table VI In essence, this is a density function of body transit times
The closed-loop transfer function is the combination of $F(t)$ and $H(t)$ according to a positive feedback control system. See Table VI. From this equation, we derive the overall body transfer function by normalizing so that it, in fact, becomes a density function of residence times. The steady-state error of this positive feedback control system equals $1/E$, so that the error is larger when the extraction is smaller. Needless to say, if the error is zero, the positive feedback system blows itself up.

1.8.3 C. The Total Body Transport Function $\Psi(t)$

The total body transfer function is essentially a normalized positive feedback transfer function. We prefer not to include the cardiac output in the overall transfer function. As remarked, it is a normalized function obtained by multiplying the closed-loop transfer function with the extraction ratio $E$ and at the same time multiplying the cardiac output in the numerator with $E$. The reason for this is that the so-called steady-state error constant of the feedback equation is $1/E$. For definition of error constants in feedback systems, see d'Azzo and Houpis. Using the definition of the clearance ($\dot{V}_{el} = \dot{V}_B \cdot E$), the overall positive feedback transfer function of Table VI can be rewritten and transformed back to the time domain. See Tables V and VI.

The overall body transport function has the dimensions of a statistical density function and can be defined as a frequency distribution of residence times. The concentration in the aorta is the convolution of input function and this body transport function $\Psi(t)$. See Table V.

The body transport function can be calculated by deconvolution of the concentration (output) curve with the input function if the latter is known. As mentioned before, in case of pulse injection directly into the vena cava, all molecules enter the system at once. Consequently the output concentration is directly proportional to the transport function. The proportionality factor is $(dose)/\dot{V}_{el}$, which equals the area under the concentration curve. So, from plasma concentration curves following a rapid bolus injection, the body transport function can be obtained by dividing the concentration data by the AUC. An example will be given in figure 14.

Often plasma concentration profiles following i.v. administration can be fitted to a sum of (1, 2, or 3) exponentials, so that then also the transport function can be described by a sum of exponentials. It should be realized that the largest errors are made during the first minutes after injection because the first pass through the heart-lungs is very fast, while there is
FIGURE 13. Output concentration curves of drugs with the same feedforward and feedback transfer functions as those of figure 12, but with varying extraction ratios \( E \). The concentration during the first few minutes has been left out. The slope of decay of the various curves strongly depends on the extraction ratio.

FIGURE 12. (Top) Theoretical transport functions of the heart-lungs (feedforward, \( H(t) \)) and of the rest-of-the-body (feedback, \( F(t) \)), as well as the open loop feedback transport function (after one whole circulation, \((1-E) F(t)*H(t)\)).

(Middle) The forward path \( H(t) \) and the first ten recirculations which show an increasing degree of dispersion and progressive extraction. Finally the curves reduce to zero (in contrast to the corresponding curves for a feedback system without extraction, where the curves attract to a fixed value > 0, see figure 8, top right).

(Bottom) The observed concentration curve (output) is the superposition of the forward path and all the recirculations (cf. figure 8, bottom right). It is a highly damped oscillation. Irrespective of the type of \( H(t) \) and \( F(t) \) functions, the result is largely a biexponential decay curve except for the first few minutes or so.

The frequency scales (abscissas) are proportional to concentration (output) scales by a factor \( AUC \), the Area Under each Curve (see § 1.8.6.-1).
still a delay of some seconds. The plasma concentration curve should start at zero concentration in any case. Extrapolation to zero time therefore has little meaning. The so-called zero time concentration will strongly depend on the number of data points shortly after injection. In fact, it is the concentration at the modal transit time of the pulmonary system. Consequently, the calculation of the so-called central volume of distribution, as usually done in compartmental kinetics, is of little meaning.

184 Dependence of the Body Transport Function on Forward and Feedback Transfer Functions

If one 'observes' the output by looking at the concentration in the aorta, the first pass is due to dispersion in the heart-lung system (figure 12 upper panel). The second pass includes convolution of $H(t) \cdot F(t) \cdot H(t)$, while only a fraction $(1-E)$ will be seen the second time. The following passes show progressive dispersion and progressive reduction due to repetitive extraction (figure 12 middle panel). The observer in the aorta sees a cumulation of the first and next passes through the body (figure 12 lower panel). This cumulative curve relates to an overdamped oscillation. It approaches zero, because there is diminuation. Compare figure 12 (middle and lower panel) to the right panels of figure 8.

Except for the initial oscillations, the curve very much resembles a biexponential curve. In fact, the shape of the forward and feedback transfer functions is not critical with respect to the shape of the entire output curve except during the first minutes after IV injection. Consequently, pharmacokinetic experiments provide little information on the forward and feedback transfer function. The importance of recirculation in pharmacokinetics has been emphasized by Vaughan and Cutler.

185 Dependence of the Body Transport Function on the Extraction Ratio

It is obvious that if the extraction were complete, no recirculation would occur, and the output would be equal to the first pass through the heart-lung subsystem. If, on the other hand, the extraction were zero, the drug would recirculate the whole life span of the body. It is evident that the extraction ratio strongly influences the profile of the body transport func-
This influence is shown in figure 13. The extraction ratio has a greater influence than the profile of the forward and feedback transfer functions because the extraction ratio determines the average number of recirculations.

186 F Analysis of the Body Transport Function

As for the analysis of subsystems, statistical moments are the methods of choice for the analysis of the system's transport function since they are integral methods using the entire plasma concentration curve. The analysis is therefore independent of fitting data to a sum of exponentials or other polynomials.

1 Areas The areas under the plasma curve (AC or AUC) equals the areas under the input and transport functions according to the system equations (see Table V). Realizing that the area under a density function equals 1, the AUC equals the dose divided by the clearance. It is also evident that if one estimates the AUC of the first pass through the pulmonary system, the AUC then equals the dose divided by the cardiac output.

2 Mean Times The mean times are calculated from the concentration curve and equal the sum of the mean time of the dose to enter the system and the mean residence time of the drug molecules in the system.

The mean residence time is composed of the mean times to pass the pulmonary system and the mean number of recirculations ($N_{rc}$) through the entire body. The mean number of recirculations depends merely on the extraction ratio ($E$): $N_{rc} = (1-E)/E$. A mean number of recirculations of 25 means that on the average, the molecules pass the system 25 times, but some molecules may be eliminated already at the first pass while others may make more than 100 transits through the body. The mean residence time is therefore largely dependent on the mean transit time through the body and the average number of recirculations. See Tables V and VII B.

Approximately, the MRT equals the MTT divided by the extraction ratio. This approximation is correct if the extraction ratio is small, as is the case for most drugs. See Table VII C. The mean transit time of phenazon is in the order of 10 min, the extraction ratio about 0.7%, and the mean residence time about 15 h. The MTT of aminophenazon is also in the order of 10 min, but its extraction ratio is about 7%, and therefore its MRT is about 5 h.
Table VII A
PHARMACOKINETIC SYSTEM PARAMETERS

\[ \dot{V}_B = \text{cardiac output} \]
\[ E = \text{extraction ratio} \]
\[ V_{el} = \text{clearance} \]
\[ \text{MTT} = \text{mean body transit time} \]
\[ \text{MAT} = \text{mean absorption time} \]
\[ \text{MIT} = \text{mean input time} \]
\[ N = \text{average number of recirculations} \]
\[ N_{rc} \]
\[ V_{dss} = \text{volume of distribution} \]
\[ \text{MRT} = \text{mean body residence time} \]
\[ h_a = \text{bioavailability (absorbed fraction)} \]

1.9 PHARMACOKINETIC SYSTEMS PARAMETERS

Irrespective of the fitting procedures used on the plasma concentration curve, the area under the curve (AUC) and the area under the time-concentration curve (TAUC) can be obtained either by applying numerical integration methods or by analytical integrations to which the data have been fitted. In fact, the AUC and TAUC are analogous to the statistical moments used to characterize statistical distribution functions.

When the drug is given by i.v. or pulse injection, the plasma concentration is directly proportional to the body transport function or density function of residence times. The moment calculations applied to the plasma concentration curve is therefore a logical way of calculating kinetic systems parameters. See Tables V and VII B. The total body clearance is directly related to the dose and to the AUC. The mean residence time is by reasonable approximation equal to the quotient of TAUC and AUC.

The cardiac output in general cannot be obtained simultaneously, but could be obtained with conventional methods such as thermodilution or i.v. indicator dilution methods. The recently developed noninvasive laser Doppler methods and NMR techniques allow cardiac output determination on a larger scale. This will be very important for a better understanding of pharmacokinetics. For the moment, simultaneous cardiac output and pharmacokinetic measurements are lacking. The combined methods would also deepen the insight into the pathology of the circulation for which the MTT is a good indicator.

If the cardiac output is known, the extraction ratio and the body mean transit time can be found. The steady-state volume of distribution is approximately the product of clearance and mean residence time.
RELATIONSHIPS BETWEEN PHARMACOKINETIC SYSTEM PARAMETERS

By definition \( \dot{V}_e = E \dot{V}_B \), \( V_{dss} = \text{MTT} \dot{V}_B \), \( N_c = (1-E)/E \)

Approximately, if \( E \) is less than 10%:

\( \text{MRT} = \text{MTT}/E \), \( V_{dss} = \dot{V}_e \times \text{MRT} \), \( N_c = 1/E \)

System parameters derived from concentration data following an intravenous pulse injection. In general:

\[
AUC = \int_0^\infty C(t) \, dt \quad T\text{AUC} = \int_0^\infty t \cdot C(t) \, dt \\
T^2 \text{AUC} = \int_0^\infty t^2 \cdot C(t) \, dt
\]

If \( C(t) \) can be fitted to a sum of exponentials \( C(t) = \sum A_j e^{-t/\tau_j} \)

\[
AUC = \sum A_j \cdot \tau_j \\
T\text{AUC} = \sum A_j \cdot \tau_j^2 \\
T^2 \text{AUC} = 2 \sum A_j \cdot \tau_j^3
\]

If \( C(t) \) can be fitted to a sum of gamma functions (where \( k_i \in (-1, +) \))

\[
C(t) = \sum A_j \cdot \left( \frac{t}{\tau_j} \right)^{k_i} e^{-t/\tau_j} \\
AUC = \sum A_j \cdot \tau_j \cdot \Gamma(k_i + 1) \\
T\text{AUC} = \sum A_j \cdot \tau_j^2 \cdot \Gamma(k_i + 2) \\
T^2 \text{AUC} = \sum A_j \cdot \tau_j^3 \cdot \Gamma(k_i + 3)
\]

Always \( \text{MRT} = \text{T\text{AUC}}/AUC \), \( \text{VRT} = T^2 \text{AUC}/AUC - (\text{T\text{AUC}}/AUC)^2 \), \( \dot{V}_e = D/AUC \)

and provided that the cardiac output is known:

\[
E = \dot{V}_e \dot{V}_B \\
\text{MTT} = E \times \text{MRT} \\
\psi(t) = C(t)/AUC
\]

The system parameters of a number of drugs have been given in Table VII C. The shortest mean body transit time is found to be in the order of 1 min. The MTT correlates with the volume of distribution. Drugs having a large volume, as many psychopharmacological agents, also have a relatively long MTT of 0.5 h (e.g., nicotine, codeine, methadon) For most drugs, the mean residence time is many hours because of a considerable number of recirculations.
### Table VII C

**PHARMACOKINETIC SYSTEM PARAMETERS OF SOME DRUGS IN MAN**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( V_{el} ) 1/h</th>
<th>( \text{MRT} ) h</th>
<th>( V_{dss} ) l</th>
<th>( E ) %</th>
<th>( \text{MTT} ) min</th>
<th>( N_{rc} )</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorouracil</td>
<td>65</td>
<td>0.15</td>
<td>9.8</td>
<td>22</td>
<td>1.8</td>
<td>3.5</td>
<td>25</td>
</tr>
<tr>
<td>Warfarin</td>
<td>32</td>
<td>40</td>
<td>12</td>
<td>0.16</td>
<td>2.6</td>
<td>624</td>
<td>26</td>
</tr>
<tr>
<td>Sulfinpyrazone</td>
<td>17</td>
<td>1</td>
<td>6</td>
<td>7.6</td>
<td>1.5</td>
<td>255</td>
<td>27</td>
</tr>
<tr>
<td>Thiamphenicol</td>
<td>42</td>
<td>2.6</td>
<td>24</td>
<td>5.3</td>
<td>4.9</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>Theophylline</td>
<td>54</td>
<td>7.9</td>
<td>2.2</td>
<td>1.16</td>
<td>5.6</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
<td>Prazosin</td>
<td>10</td>
<td>2.8</td>
<td>2.9</td>
<td>3.47</td>
<td>5.8</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Phenazon</td>
<td>2.17</td>
<td>14</td>
<td>5</td>
<td>32</td>
<td>0.7</td>
<td>6.3</td>
<td>142</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>22</td>
<td>1.24</td>
<td>4.4</td>
<td>7.6</td>
<td>8.8</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>Propranolol</td>
<td>43</td>
<td>1.9</td>
<td>8.2</td>
<td>14</td>
<td>16</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>19</td>
<td>3.2</td>
<td>6.3</td>
<td>6.4</td>
<td>12</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>Phencetin</td>
<td>66</td>
<td>1.4</td>
<td>8.9</td>
<td>22</td>
<td>15</td>
<td>3.5</td>
<td>35</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>14</td>
<td>5.4</td>
<td>7.5</td>
<td>4.7</td>
<td>25</td>
<td>20</td>
<td>36</td>
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<tr>
<td>Nicotine</td>
<td>77</td>
<td>2.4</td>
<td>18.3</td>
<td>2.5</td>
<td>36</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>Pindolol</td>
<td>18</td>
<td>2.9</td>
<td>5.5</td>
<td>6.3</td>
<td>11</td>
<td>15</td>
<td>38</td>
</tr>
<tr>
<td>Butobarbital</td>
<td>1.08</td>
<td>5.4</td>
<td>58</td>
<td>0.36</td>
<td>12</td>
<td>275</td>
<td>39</td>
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<tr>
<td>Lorazepam</td>
<td>11</td>
<td>4</td>
<td>10</td>
<td>5.120</td>
<td>3.5</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>Caffeine</td>
<td>7.15</td>
<td>6.5</td>
<td>4.6</td>
<td>2.0</td>
<td>7.7</td>
<td>50 *</td>
<td></td>
</tr>
<tr>
<td>Dexamphetamine</td>
<td>21</td>
<td>9.8</td>
<td>21.0</td>
<td>7.12</td>
<td>42</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Codeine</td>
<td>45</td>
<td>4</td>
<td>18.2</td>
<td>15</td>
<td>36</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>Meperidine</td>
<td>57</td>
<td>3.7</td>
<td>20.9</td>
<td>19</td>
<td>42</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Methadon</td>
<td>8</td>
<td>10</td>
<td>240</td>
<td>2.5</td>
<td>48</td>
<td>39</td>
<td>45</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>37</td>
<td>3.8</td>
<td>141</td>
<td>1.2</td>
<td>28</td>
<td>7</td>
<td>46</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>54</td>
<td>2.3</td>
<td>90</td>
<td>15</td>
<td>18</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>82</td>
<td>1.2</td>
<td>9.6</td>
<td>27.2</td>
<td>20</td>
<td>3</td>
<td>48</td>
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<tr>
<td>Quinine</td>
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<td>1.2</td>
<td>97</td>
<td>2.2</td>
<td>16</td>
<td>45 *</td>
<td></td>
</tr>
<tr>
<td>Cotinine</td>
<td>3.6</td>
<td>14</td>
<td>5.2</td>
<td>1.0</td>
<td>8.7</td>
<td>99 *</td>
<td></td>
</tr>
</tbody>
</table>

Note: The cardiac output \( V_B \) was assumed to be 6 l/min.

* This thesis.

## 1.10 CALCULATION OF THE BODY TRANSPORT FUNCTION

The profile of the body transport function \( \psi(t) \) can be calculated from the plasma concentration, provided that the input function is known, and the transport function from application site to mixed venous pool (see §1.11) may
be neglected. The latter is obviously the case for \( i.v. \) injections directly into the vena cava, but also for injections in an arm vein except for drugs with very fast dynamics.

Since the output concentration is a convolution of input function and body transport function, the latter can be found by the inverse operation deconvolution:

\[
\psi(t) = \frac{\dot{V}_{el}}{V_c} C(t) \cdot D(t)^{-1} \quad \text{or} \quad \psi(s) = \frac{\dot{V}_{el}}{V_c} C(s) / D(s)
\]

In general, the transport function can be obtained by a numerical deconvolution procedure either by using matrix division methods or by first transforming \( C(t) \) and \( D(t) \) into the Laplace or Fourier domain, dividing the functions \( C(s)/D(s) \), and then transforming the result back to the time domain. Several numerical deconvolution methods have been reported.

If the input function is the Dirac delta pulse, the output is directly proportional to the transport function. Consequently, the inverse operation deconvolution is equal to division. This implies that in case of \( i.v. \) injection of the drug, its transport function in the injected volunteer can be calculated by dividing the observed concentration vs. time profile by the area under it (AUC).

If the concentration vs. time profile after \( i.v. \) injection of the drug can be described satisfactorily by an analytical expression, a similar analytical expression is easily derived for \( \psi(t) \). For example, if the profile can be fitted to a sum of exponentials,

\[
C(t) = \sum_{i=1}^{m} A_i \cdot e^{-t/\tau_i},
\]

then the body transport function \( \psi(t) \), by definition, is found as

\[
\psi(t) = \frac{\sum_{i=1}^{m} A_i \cdot e^{-t/\tau_i}}{\sum_{i=1}^{m} A_i \cdot \tau_i}.
\]

If the same dose of the drug with the above \( \psi(t) \) were to be administered by constant \( i.v. \) infusion over a period \( T \) to the same volunteer, it can be shown that its concentration vs. time profile during and after the infusion would fit to

\[
C(t) = \sum_{i=1}^{m} \left( \frac{A_i \cdot \tau_i}{T} \right) \cdot (1-e^{-T/\tau_i}) \cdot e^{-t/T} \cdot e^{-(t-T)/\tau_i}, \quad \text{and}
\]

\[
C(t) = \sum_{i=1}^{m} \left( \frac{A_i \cdot \tau_i}{T} \right) \cdot (1-e^{-T/\tau_i}) \cdot e^{-t/T} \cdot e^{-(t-T)/\tau_i}.
\]
respectively. Hence \( \psi(t) \) is also computable from the concentration vs. time profile during and following constant \( iv \) infusion of the drug. The results of this procedure in calculating \( \psi(t) \) of quinine in a healthy female volunteer is shown in Figure 14.

**FIGURE 14** (Top left) Venous concentration curve of quinine following a known input (here \( iv \) infusion) in a human subject. (Bottom left) Total body transport function of quinine as calculated from the upper profile. (Top right) Venous concentration curve in the same subject following an unknown input via the GI tract after ingestion of a capsule containing a quinine salt. (Bottom right) Input function from GI tract into the venous pool calculated from the output following oral dosage and the total body transport function. (Reproduced from Teeuwen, H W A et al., in preparation)
As the overall body transport function is the characteristic function, fully dominating what the output will be for a given input, knowledge about the transport function is important in order to calculate what the output will be when various inputs are offered (convolution), or it can be used to calculate the input function giving rise to an observed output (deconvolution).

### 111.1 Input Functions at the Application Site

In general the input function \( D(t) \) is not offered directly to the mixed venous pool, but at some application site which may be a peripheral vein, the skin, the GI tract, a muscle, the respiratory tract or any other location. This implies that a transport function \( H_a(t) \) has to be included in the systems equation which determines the transport from application site to

A realistic block diagram of the body transport function with input not into the venous pool but rather at an application site, and with output observation not in the aorta but rather in a peripheral vein. Two additional transport functions \( H_a(t) \) and \( F_o(t) \) are involved. See also figure 11 and Table VIII.

The area under this function \( AH_a \) may be less than unity if some drug is lost during this transport (incomplete absorption). In fact, \( AH_a = h_a \) is the biological availability. It will also take some time to pass the \( H_a(t) \) function as indicated by a mean absorption time MAT.
The output is, in general, not measured in the aorta but in a peripheral vein or at some other observation site. See figure 15. Therefore, again a transport function $F_0(t)$ has to be included describing the transport from aorta to this observation site. Often this function is very fast with respect to the total body transport function $\Psi(t)$, so that it may be neglected.

The general systems equation, then, has to be extended with $H_a(t)$ and $F_0(t)$, but the principles as discussed before remain valid. See Table VIII.

1112 B. The Intravenous Input Function

If drugs are not injected into the vena cava, but into a vein of the arm, the application transport function $H_a(t)$ is short. A pulse input function is, however, already dispersed or distorted to some extent when the drug reaches the the vena cava or mixed venous pool. The additional transport function governing transport from injection site to mixed venous pool has no influence on the positive feedback body transport function, except that when very fast processes are observed, as in the first passes through the heart-lungs, these may cause great errors in the output.

During passage from injection site to mixed venous pool, in general no drug is lost ($h_a = 1$) so that from the analysis by statistical moments, the clearance still can be calculated. The mean time from injection site to venous pool is in the order of seconds, so that this transport function often may be neglected. Consequently, the overall body transport function can be obtained most easily from IV drug application.

The input functions usually applied by the IV route are the pulse or bolus injection and the step function or constant IV infusion.

**Pulse injection** The output concentration is directly proportional to the convolution of $H_a(t)$, $\Psi(t)$ and and $F_0(t)$, or approximately to $\Psi(t)$ alone, neglecting $H_a(t)$ and $F_0(t)$. This approximation is certainly allowed for the plasma concentration with a large mean residence time, but large errors may occur for the fast time constants of the curve, that is, just after injection.

**Intravenous infusion** The output concentration is the integral of the convolution of $H_a(t)$, $\Psi(t)$, and $F_0(t)$. Since $H_a(t)$ and $F_0(t)$ may be neglected, in fact, the output is simply the integral of the transport function $\Psi(t)$. If the infusion is continued long enough, a steady-state output concentration is obtained since the area under $\Psi(t)$ is one. See Table VIII.
Table VIII
SYSTEMS DYNAMICS OF DRUG APPLICATION IN GENERAL

Systems equation

Laplace s-domain
\[ C(s) = \frac{D_a(s)}{V_B} \cdot H_a(s) \cdot \frac{H(s)}{1 - (1 - E) \cdot F(s) \cdot H(s)} \cdot F_0(s) \]
\[ C(s) = \frac{D_a(s)}{V_{el}} \cdot H_a(s) \cdot \psi(s) \cdot F_0(s) \]

Time domain
\[ C(t) = \frac{D_a(t)}{V_{el}} \cdot H_a(t) \cdot \psi(t) \cdot F_0(t) \]

Analysis

Areas
\[ AC = \frac{AD}{V_{el}} \cdot A_H \cdot A\psi \cdot AF_0 = \frac{\text{dose} \cdot h_a}{V_{el}} \]

Mean times
\[ TC = TD + TH_a + MRT + TF \]
\[ TC = \text{MIT} + MRT \]

Intravenous input functions
\[ H_a(t) + H_i(t) \quad \text{and} \quad h_a = 1 \]

a) Pulse or bolus \((D(t) = D \delta(t))\)
\[ C(t) = \frac{D}{V_{el}} \cdot H_i(t) \cdot \psi(t) = \frac{D}{V_{el}} \cdot \psi(t) \]

b) Step function or constant rate infusion \((D(t) = \dot{D})\)
\[ C(t) = \frac{\dot{D}}{V_{el}} \int_0^t \psi(\lambda) \cdot d\lambda \]

Steady state \((t \to \infty)\)
\[ Css = \frac{\dot{D}}{V_{el}} \]

Note: \(\delta(t)\) = the Dirac delta pulse function, \(\dot{D}\) = the constant step function input rate (mg/h)

@ Input drug dosage flow at application site Output drug dosage flow along observation site = concentration in the blood at observation site times the blood flow

From concentration curves during and after infusion, it is relatively easy to calculate the body transport function. See figure 15 and the previous paragraph.
Inhalation of drugs is a common practice in anaesthesiology and in the treatment of asthma and bronchitis. In addition, smoking of tobacco is aimed at input of nicotine via the lung alveoli.

The bronchi receive blood from the general circulation and deliver back to the general circulation. The alveoli are in the pulmonary circulation, while the bronchioli receive blood from the left heart and deliver blood to the pulmonary circulation. This implies that drugs applied to the respiratory tract may reach different parts of the circulation via two major routes (figure 16). The relative importance of the two routes largely depends on the physico-chemical properties of the drug (e.g., state of aggregation), its pharmaceutical formulation (e.g., dispersion grade of aerosols) and on the depth and duration of inhalation.

In general, a fraction $f_A$ is absorbed in the alveoli and small bronchioli and transported to the aorta, this absorption and subsequent transport is characterized by the transport function $H_{LA}(t)$. The absorption via the alveoli may be very rapid, such that drugs given to the lungs may reach the aorta even faster than following IV injection due to bypass of the $H(t)$ transport function (figure 16).

Another fraction $f_B$ may be absorbed relatively slowly via the trachea, the bronchi and the bronchioli, this absorption and transport being characterized by the function $H_{LB}(t)$. Consequently, the overall transport
function $H_L(t)$ from application site to venous pool contains two terms. The overall systemic availability $h_a$ equals the summation of $f_A$ and $f_B$ (in this thesis, $f_L = f_A + f_B$ will be the symbol of total systemic availability of nicotine in the respiratory tract, reserving $h_a$ as the symbol for GI tract systemic availability).

The nicotine intake by cigarette smoking mainly occurs via the lung alveoli since the major part of nicotine in cigarette smoke is associated to aerosols, and since the particle size of cigarette smoke aerosols in general is very small (about a few tenth of a µm on the average). The complementary (minute) part of nicotine in cigarette smoke is vaporized and is deposited completely in the deep airways. The transport function $H_{LA}(t)$ is very fast for nicotine; indeed an inhaled dose of nicotine reaches the aorta (and the brain) faster (7 sec) than a dose injected in an arm vein (14 sec). It is argued that habituation to nicotine is a dependence on high nicotine bolus in the brain. If this is true, cigarette smoking is able to produce habituation by the grace of the low-dispersion respiratory tract transport function. Almost any other route of nicotine administration (including the oral route used in case of nicotine chewing gum) would be characterized by slower dynamics and consequently would produce more dispersed low-peaked nicotine bumps in the cerebral blood vessels.

Cigarettes are smoked taking a number of puffs in rapid succession (ca. 10 per cigarette), while the interval between the cigarettes may vary considerably. As the dynamics of the application transport function $H_{LA}(t)$ are rapid as stated before, the nicotine concentration in the blood (the output) rises with every puff of cigarette smoke (the input or dosage). Since the body transport function of nicotine (governing nicotine disposition) is very fast as well, the output rapidly decreases if the input is zero for a while. So, nicotine output follows nicotine input, and there is a rise and fall in the nicotine concentration with every cigarette. See figure 17.

The time course of nicotine's principle metabolite cotinine however is heavily damped due to the slow disposition kinetics of this metabolite, once formed. The metabolic input (formation) function is dealt with in § 1.13.
FIGURE 17. Plasma concentration curves of (o) the parent drug nicotine and (*) its major metabolite cotinine over 24 h in a regular steady smoker. Every arrow denotes the (time of) smoking of a single cigarette. The input- and transport functions of nicotine are both fast, and consequently, nicotine output rapidly follows its input. On the other hand, the transport function of cotinine is very slow. Therefore cotinine output does not follow nicotine input, but rather averages it. (From Teeuwen H.W.A., unpublished data).

1.12 ANALYSIS OF INPUT FUNCTIONS AND APPLICATION SITE TRANSPORT FUNCTIONS

Once the body transport function is known by calculation from a measured output following a known i.v. input, unknown input functions can also be calculated as well as the transport function from application site to venous pool. The general equation is (see Table IX):

\[ C(t) = \left(\frac{1}{V_{el}}\right) D(t) \ast H_a(t) \ast \psi(t) \ast F_o(t) \]
So, if $\psi(t)$ is known, $D(t)H_a(t)$ can also be obtained. An example is given for the oral intake of a capsule of quinine in figure 14. The results show that this function is certainly not of a monoexponentially decaying type as suggested by classic compartmental pharmacokinetics. There is quite a variation in the transport rate of quinine from the GI tract to the venous pool in different individuals.

112.1 A Analysis of the Application Function $H_a(t)$

From input-output studies with known $\psi(t)$, one still does not have $D(t)$ or $H_a(t)$ separately, but rather their convolution (see figure 14, bottom right). If, however, $D(t)$ is reduced to a pulse, the general solution reduces to

$$C(t) = \left(1/V_{el}\right) D \ast H_a(t) \ast \psi(t)$$

This implies that $H_a(t)$ then can be calculated from the output concentration, provided that $\psi(t)$ has been determined using (analytical or numerical) deconvolution. The analysis is summarized in Table IX using Laplace transforms.

Examples of momentary (pulse) input functions are the intramuscular injection of a small volume solution, or the oral administration of a solution of a drug. In both examples the drug is brought practically directly without losses to the application site, where the function $H_a(t)$ takes over.

112.2 B Analysis of the Input Function by Eliminating the Transport Function

The input function of an oral tablet or capsule can be calculated by a deconvolution operation on the output concentration of that tablet and a solution of the drug. This implies that in the two experiments, the $H_a(t)$ is expected to remain the same. In essence, the $H_a(t)$ is not separated from $\psi(t)$. So, by measuring the output of a solution, one calculates $H_a(t)\ast\psi(t)$. A deconvolution operation on the output following the tablet using the previously found $H_a(t)\ast\psi(t)$ directly gives $D(t)$.

If one measures the output concentration following the oral intake of a tablet and a retard preparation, obviously deconvolution does not give the...
Table IX

ANALYSIS OF APPLICATION TRANSFER FUNCTIONS AND INPUT FUNCTIONS

A. Analysis of the application transfer function \( H_a(s) \).

Systems equation (Laplace domain);

after pulse intravenous input · after pulse input at application site:

\[
C_i(s) = D_i(s) \cdot \frac{H_i(s)}{V_{el}} \cdot \psi(s) \cdot F_o(s) \\
C_a(s) = D_a(s) \cdot \frac{H_a(s)}{V_{el}} \cdot \psi(s) \cdot F_o(s)
\]

where \( D_i(s) = D, H_i(s) = 1 \), and \( D_a(s) = D_a \).

Intra-individual variability of \( V_{el}, \psi(s), \) and \( F_o(s) \) is neglected.

Analysis of \( H_a(s) \) from i.v. input and pulse input at application site:

\[
H_a(s) = \frac{C_a(s)}{C_i(s)} \cdot \frac{D}{D_a}
\]

bioavailability: mean absorption time:

\[
\text{AUC}_a \cdot D_a \\
\text{AUC}_i \cdot D_i
\]

\( \text{TH}_a = T_C - T_C_i \)

B. Analysis of the input function \( D_{a,ret}(s) \) of a retard preparation.

Systems equation (Laplace domain)

after pulse input of a solution \( (D_{a,sol}(s) = D_{a,sol}) \):

\[
C_{a,sol}(s) = D_{a,sol}(s) \cdot \frac{H_a(s)}{V_{el}} \cdot \psi(s) \cdot F_o(s)
\]

after input of a particular retard preparation:

\[
C_{a,ret}(s) = D_{a,ret}(s) \cdot \frac{H_a(s)}{V_{el}} \cdot \psi(s) \cdot F_o(s)
\]

Intra-individual variability of \( V_{el}, \psi(s), F_o(s) \) and \( H_a(s) \) is neglected.

Analysis of \( D_{a,ret}(s) \) from a dose as a solution and a dose as a retard preparation.

\[
D_{a,ret}(s) = \frac{C_{a,ret}(s)}{C_{a,sol}(s)} \cdot D_{a,sol}
\]

absorbed dose. mean dissolution time:

\[
\text{TD}_{a,ret} = T_C_{a,ret} - T_C_{a,sol}
\]

as \( \text{TD}_{a,sol} = 0 \)

input of the retard preparation. It does, however, provide a good measure of the relative performance of the two formulations. Again it is not necessary to know the body transport function nor the application site transport function as may easily be concluded from the systems equations in the Laplace domain. See Table IX.
It must be realized that it is not always possible to perform the analysis since it may occur that the application transport function is different for the two pharmaceutical dosage forms that are compared. For instance, a solution may pass immediately from stomach to duodenum, whereas the retard tablet may distribute over a different part of the GI tract.

1.13 THE METABOLIC INPUT FUNCTION

Most drugs are transformed in the body into metabolites which may have pharmacological activity. With regard to a metabolite concentration profile, the parent drug concentration is input to the metabolite transport function. See figure 18. The system equation for the parent drug and metabolite is given in Table X. It is evident that the two transport functions are in series. Consequently, the metabolite curve is more dispersed than the concentration curve of the parent drug.

Three types of interrelationship between the transport functions of parent drug and metabolite may be discerned:

- If the dynamics of the metabolite is much faster than the dynamics of the parent drug, there is practically no further dispersion and the profile of the metabolite is identical to that of the parent drug. An example is the kinetics of trichloroethanol and its glucuronic acid metabolite.

- The dynamics of the metabolite is much slower than the dynamics of the parent drug. Then the dispersion by the metabolite is so large that the curve of the parent drug may be considered as a pulse. The profile of the metabolite curve is very much flatter. An example is found in nicotine and its major metabolite cotinine. As we saw in §1.11 C, in cigarette smoking, nicotine output follows its input practically without delay as a consequence of the fast dynamics of the application transport function $H_L(t)$ (governing alveolar nicotine absorption) and the body transport function $\psi(t)$ (governing nicotine disposition). There is a rise and fall in the nicotine plasma concentration with every individual cigarette smoked (see figure 17). The body transport function of cotinine, however, is slower so that the cotinine output concentration cannot follow the input variation of nicotine. Instead it averages the intake variation of nicotine. In this respect, the cotinine plasma level is a valuable tool to estimate the daily intake of its parent nicotine in tobacco smokers.
FIGURE 18 Feedback diagram for the parent drug and its major metabolite. When observing the metabolite concentration, the concentration (output) of the parent drug acts as input to the metabolite system, operated on by the transport function of the metabolite. Hence, the two transport functions (of parent and metabolite) are arranged in series. See Table X

- The dynamics of the parent drug and the metabolite are very much the same. The metabolite curve is more, but not much more dispersed than the curve of the parent drug, and assumes a typical convex 'gamma' shape. After approximately two times the common MRT of both compounds, the curves of parent and metabolite roughly parallel each other. An example is found in caffeine and its main metabolite paraxanthine (figure 19)
Table X
CONTROL SYSTEMS DYNAMICS OF DRUG METABOLISM

Systems equation
In Laplace s-domain:
\[ C_m(s) = D_a(s) \cdot \frac{H_a(s)}{V_B} \cdot \frac{H(s)}{1-(1-E) \cdot F(s)H(s)} \cdot E \cdot f_m \cdot F(s) \cdot \frac{H_m(s)}{1-(1-E_m) \cdot F_m(s)H_m(s)} \cdot F_{0m}(s) \]
\[ C_m(s) = D_a(s) \cdot \frac{H_a(s)}{V_{mel}} \cdot \psi(s) \cdot F(s) \cdot \psi_m(s) \cdot F_{0m}(s) \]

where
\[ \psi(s) = \frac{E \cdot H(s)}{1-(1-E) \cdot F(s)H(s)} \quad \text{and} \quad \psi_m(s) = \frac{E_m \cdot H_m(s)}{1-(1-E_m) \cdot F_m(s)H_m(s)} \]

In time domain:
\[ C_m(t) = D_a(t) \cdot \frac{H_a}{V_{mel}} \cdot \psi(t) \cdot f_m \cdot F(t) \cdot \psi_m(t) \cdot F_{0m}(t) \]

Analysis
Areas:
\[ A_{C_m} = AD_a \cdot \frac{AH_a}{V_B} \cdot \frac{AH \cdot AF \cdot E}{1-(1-E) \cdot AF \cdot AH} \cdot f_m \cdot \frac{AH_m \cdot AF_{0m}}{1-(1-E_m) \cdot AF_m \cdot AH_m} \]
\[ A_{C_m} = \text{dose} \cdot f_a \cdot f_m \]

Mean times:
\[ T_{C_m} = T_A + TH + T_T + TF + T_{T_m} + TF_{0m} \]

where:
\[ T_T = MRT + TH + \frac{1-E}{E} \cdot MTT \quad \text{and} \quad T_{T_m} = MRT_m + TH_m + \frac{1-E_m}{E_m} \cdot MTT_m \]

approximately:
\[ T_{C_m} = MAT + MRT + MRT_m \]

1.14 CONCLUSIONS

The body is a dynamical system whose future state depends on its present state and inputs to the system. In pharmacokinetics, only a limited number of state variables are sufficient to characterize the system behavior. In general, point attractors dominate the kinetics.

The various organs and tissues may be considered as subsystems. Their kinetic behavior is fully characterized by density functions of transit times and their bloodflow.
Table X (continued)

CONTROL SYSTEMS DYNAMICS OF DRUG METABOLISM

Special input functions of the parent compound

1) i.v pulse injection ($D(t) = D\delta(t)$, $h_a = 1$, $H_a(t)$ fast)

$$C_m(t) = \frac{D}{V_{el}} \cdot \psi(t) \cdot f_m \cdot F(t) \cdot \psi_m(t) \cdot F_{mo}(t)$$

2) i.v constant rate infusion ($D(t) = D\dot{t}$, $h_a = 1$, $H_a(t)$ fast)

$$C_m(t) = \frac{D}{V_{mel}} \int_0^t \psi(t) \cdot F(t) \cdot \psi_m(t) \cdot F_{mo}(t) \cdot dt$$

Steady state ($t\rightarrow\infty$)

$$C_m(\infty) = \frac{D \cdot f_m}{V_{mel}}$$

Note. All metabolite parameters bear a subscript "m" if it is the fraction of the parent compound converted into the particular metabolite

@ Parent drug (P) input P dosage flow at application site

P output P dosage flow along the observation site = P blood conc times the blood flow

Metabolite (M) input metabolic conversion rate of P into M, dosage flow of M out of the converting organs and tissues

M output M dosage flow along the observation site = M blood conc times the blood flow

The total body is an arrangement of the subsystems according to a positive feedback control system. The open-loop feedback transport function is a density function of body transit times, while the closed-loop transport function is, in essence, a density function of residence times.

The important pharmacokinetic system parameters are the mean body residence time, the cardiac output, the extraction ratio, the total clearance, and the apparent volume of distribution.

The overall body transport function can be found from measuring the output concentration when applying a known input function using deconvolution methods. Unknown dosage input functions to the body, either by oral intake, intramuscular injection, inhalation or otherwise, can be found from measuring the output concentration of such an unknown input after first obtaining the body transport function.

Since the body transport function is fixed for a given individual and a given drug in a short period of time, a required output can only be obtained
FIGURE 19 (Top left) Venous concentration curve of paraxanthine (main metabolite of caffeine) following a known input (here iv infusion) in a human subject (Bottom left) Total body transport function of paraxanthine as calculated from the infusion profile (Top right) Venous concentration curve in the same subject following an unknown metabolic input after an iv dose of the parent compound caffeine. The venous caffeine concentration curve is shown as well (Bottom right) Metabolic input function of paraxanthine by caffeine biotransformation, as calculated from the output following caffeine dosage and the paraxanthine transport function. Note the analogy with figure 14 (Reproduced from Teeuwen, H W A et al., in preparation)

by devising the correct input function. Computational experiments may be helpful to design the adequate dosage form and schedule to fulfill such requirements.
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SECTION II

CHROMATOGRAPHY-BASED ANALYTICAL METHODS
Chapter 2

SIMULTANEOUS ESTIMATION OF NICOTINE AND COTININE
LEVELS IN BIOLOGICAL FLUIDS USING HIGH-RESOLUTION
CAPILLARY-COLUMN GAS CHROMATOGRAPHY COMBINED WITH
SOLID PHASE EXTRACTION WORK-UP

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2.1 SUMMARY

A rapid and sensitive capillary gas-chromatographic method with
nitrogen-sensitive detection is reported for the simultaneous analysis of
nicotine and cotinine levels occurring in the plasma, saliva, and urine of
regular tobacco smokers. The proposed assay has a linear output, has satis­
factory accuracy over the range of concentrations of both amines encountered
in active smokers, and has also been successful in the analysis of the urine
samples of passive smokers. Its lower limit of sensitivity is 0.2 ng of nico­
tine and 0.5 ng of cotinine per ml of plasma or saliva or per 100 ml of urine.

The beneficial characteristics of the presented method were achieved by
the combination of solid phase extraction of 0.1 - 1.0 ml of fluid specimens,
capillary column gas chromatography with splitless injection and nitrogen sen­sitive detection, and the use of separate, structurally analogous compounds as
internal standards for nicotine and cotinine. The suitability of the assay is
shown by plasma concentration-time curves of nicotine and cotinine in a steady
smoker during a 24 hours period.
Concomittant with the increasing public awareness of the relationship existing between the active intake, and maybe the passive intake as well, of tobacco and tobacco smoke constituents, and the chance of developing a tobacco-use related disease, the measurement of nicotine and its major phase-I metabolite cotinine in body fluids has gained increased attention in the past two decades.

Nicotine is the most abundant and the most potent pharmacological agent in tobacco and tobacco smoke. It is the primary, though not the only reason why people consume tobacco and it may contribute to causation of some of the diseases that are related to the use of tobacco requisites. The nicotine delivery of the cigarette to the smoker must be the limiting factor in the rate of nicotine self-administration by smokers [1]. While regulating his nicotine input rate, the smoker at the same time co-regulates the input rate of the smoke constituents other than nicotine present in both the particulate phase (water, 'tar') and the gaseous phase (e.g., carbon monoxide) of the smoke.

Methods reported for the assay of nicotine and cotinine in biological material cover a large field of analytical techniques. A qualitative mass spectrometrical assay has been published by Horning et al. [2]. Radioimmunoassays [3-11] and a related procedure [12] have been reported but are very cumbersome and therefore inappropriate when large amounts of samples are to be analyzed. High-performance liquid chromatographic techniques employing detection by UV-absorbance [13-15] typically are not sensitive enough to cover the range of concentrations below approximately 50 ng/mL of sample, so their use is restricted to urine samples of active smokers.

Gas chromatographical (GC) methods on the other hand can be rendered very rapid and sensitive. A large variety of GC methods has been reported for the analysis of nicotine and cotinine in biological fluids using flame ionization detection (FID) [16-19], electron capture detection (ECD, after derivatization) [20], nitrogen phosphorous detection (NPD) [21-29], and detection by mass spectrometry combined with the facility of selected ion monitoring (MS/SIM) [29-33].

In the analysis of nicotine and cotinine by GC, the following parameters must be tuned deliberately: sample volume, extraction procedure, choice of internal standards, type of column, injection mode, isothermal or temperature-programmed elution, and sensitivity and specificity of the detector. These
Important considerations will now be discussed in some detail while comparing published methods.

Sample volume. Except for urine, the available sample volume in general is limited, especially so when the sample source is a baby or a small laboratory animal, or when repetitive sampling from a single subject is intended, e.g., in the course of a pharmacokinetic experiment. Reported methods require non-urine sample volumes that range from 10 ml [30] to 100 μl in the micromethods developed by Feyerabend and Russell, and Curvali et al. [23, 27], or even to 50 μl [32]. Most methods require 1 ml of plasma [17, 19, 22, 25-28, 33].

A comparable quantity is the requirement of 1 g of tissue by Thompson et al. [29].

Extraction. An extraction procedure should recover nicotine and cotinine simultaneously and completely from plasma, urine, and other relevant biological materials in a single and rapid extraction step, at the same time separating the two amines and their internal standards from the bulk of the potentially interfering endogenous fluid constituents in order to create beneficial conditions for the subsequent chromatographical run. Unfortunately, the vast majority of the published nicotine and cotinine assays involve extraction procedures that fail to meet one or more of these criteria.

The most commonly encountered handicap is a multiplicity of liquid extraction steps that can grow to unworkable proportions. Three reasons may be conceived to introduce more than one extraction. First, analyte recovery may be enhanced by extracting a sample with a few small portions of extraction solvent, rather than extracting it with one big portion [16, 19, 21, 22, 30-32].

Second, extraction of the alkaloids and their standards in an organic solvent under alkaline conditions may be preceded or followed by an acidic extraction of the sample to remove weakly acidic or neutral interferences [19, 20, 22, 23, 25, 26, 29, 31, 32]. In case the acidic extraction follows the alkaline one, a third extraction is needed to finally transfer nicotine and cotinine into a non-aqueous milieu, suitable for evaporative concentration and injection into a gas chromatograph. In spite of their complexity, the degree of clean-up realized with these acidic-alkaline or alkaline-acidic-alkaline extractions is likely to be questionable, witness the fact that the authors of only two assays involving these extraction sequences stated that their assay was fit to urine analysis [23, 26].
The third reason for the performance of more than one liquid extraction step is the separate extraction of nicotine and cotinine in those methods claiming to be applicable to the analysis of both amines, using different extraction solvents \[16, 26, 22, 29\]. Most frequently, nicotine and cotinine are extracted in diethyl ether and dichloromethane, respectively. Dichloromethane appears to be the only solvent in which both nicotine and cotinine may be extracted simultaneously and to a good extent. Kogan et al., Curvall et al., and Davis [25, 27, 28] utilized it as an extraction solvent, combining it with NPD. Kogan cum suis [25] employed a packed column and a three-step plasma extraction procedure of the alkaline-acidic-alkaline type. For Curvall and co-workers [27] and Davis [28] who employed capillary columns, a single-step extraction sufficed. Neither of these three assays was stated to be suited to urine analysis by the authors.

It is obvious that multiple extraction steps may give rise to low recoveries of the analyte(s) and so to reduced sensitivity and accuracy of the assay. Compensation for the extraction losses may be found in bulky sample volumes \[16, 19, 21, 23, 30, 31\]. In addition, lengthy extraction procedures are counterproductive and promote interference from extraneous nicotine as stated by Curvall et al. [27].

As an alternative, Stehlik et al. [18] and Kyerematen et al. [15] isolated nicotine and cotinine from biological materials by making use of selective adsorption of both amines onto the stationary phase of a disposable extraction column. This type of extraction is known as 'solid phase' extraction (SPE). SPE in our laboratory has proven to be a very powerful extraction technique. In accordance with our experience, both Stehlik and Kyerematen succeeded in extracting nicotine and cotinine simultaneously, with good recovery and with a sufficient degree of clean-up in a single extraction step from urine. The extraction procedure of Kyerematen can be applied without modifications to plasma as well, but that of Stehlik is modified for plasma and involves three steps. We set it our goal to develop a SPE-procedure applicable without any modifications to the GC-analysis of nicotine and cotinine in both plasma and urine.

Internal standards. Jacob et al. [26] were critical of the use of quinoline and lidocaine as internal standards (ISTDs) for nicotine and cotinine. They observed a large variability in the peak height ratio of nicotine to quinoline analyzing identical samples, and suggested that this variability was due to different relative losses of nicotine and its ISTD during sample work-
up and/or during gas chromatography. In general, they recommended the use of structural analogs as ISTDs, because their similar physico-chemical properties guarantee similar relative losses in the course of the analysis procedure.

As a consequence, dual internal standards should be used for nicotine and cotinine, for the physico-chemical properties of these two compounds differ widely.

Quinoline, chlorphentermine, ketamine, modaline, N-ethyl-nornicotine, N-n-propyl-nornicotine, N-methyl-anabasine, nicotine-5,5'-D₂, and N-3H₂C-nornicotine have been used as nicotine-dedicated ISTDs [16,18-23,26-29,31,32]. Lidocaine, lignocaine, pheniramine, N-(2-methoxyethyl)-norcotinine, 2'-oxy N-methyl-anabasine, N-ethyl-norcotinine and N-3H₂C-norcotinine have been reported as ISTDs dedicated to cotinine [16,18,22,26-29,33]. Furthermore, Kogan and co-workers employed ketamine as an ISTD for both nicotine and cotinine [25]. Falkman et al. added their 'internal' standard quinoline after extraction of nicotine [30], implicating that the standard cannot correct for analyte losses during the extraction. The authors therefore should have called their standard an external standard. Grubner, First and Huber do not use any standard at all [24].

The degree of structural analogy existing between analyte and ISTD in the various assays ranges from poor (first three nicotine and cotinine standards mentioned) to excellent (isotope-labeled analogs). However, use of these labeled analogs restricts the spectrum of applicable detection techniques to the expensive mass spectrometer.

Chromatography. Gas chromatographical separation of nicotine and/or cotinine, standard(s), and endogenous compounds evoking a detector response may be achieved with packed [16,17,19,20-26,32] and with capillary open tubular columns [18,27-29,31,33]. Also, gas chromatography may be performed isothermally [16,17,19-26,30,32] or with oven temperature programming [18,27-29,31,33].

Isothermal runs have the advantage of yielding stable base lines without noise and drift, a condition that fosters sensitivity and accuracy especially when employing packed GC-columns. For capillary GC, isothermal operation implicates split injection and a concomitant decimation in sensitivity because of the loss of typically more than 90% of the injected materials through the split vent. As a consequence, there are many assays combining isothermal operation with the use of packed columns [16,17,19-24,26,30], whereas only a few combine it with capillary columns [27,31].
Oven temperature programming on the other hand offers the advantage of eluting both nicotine and cotinine within a relatively short period of time. In temperature-programmed capillary GC, one has the option of avoiding sensitivity decimation and at the same time substantially increasing peak resolution by using the splitless injection mode. The injection conditions in this case should be chosen deliberately so as to optimally benefit by the so-called 'cold trap' and the 'solvent effect' mechanisms of analyte reconcentration in narrow bands at the head of the capillary column [34]. The chromatographical strategy in the analytical methods proposed by Thompson, Stehlik, Daenens and their respective colleagues, and by Davis [18,29,33,38] is based on the latter considerations.

All isothermal methods referred to, as well as one with temperature programming, that are reported for the analysis of both nicotine and cotinine [16,22,26,27,29] elute the two amines in parallel chromatographical runs using two separate chromatographs, each operating at the conditions specific to the determination of the individual analyte. With the exception of the Curvall method [27], these methods involve separate extraction procedures as well for nicotine and its main metabolite, so that actually these assay procedures [16,22,26,29] are composed of two distinct analytical methods that start from the same sample and employ the same type of equipment, rather than constituting truly a single and simultaneous assay. Both simultaneous extraction and chromatography is accomplished only by Davis [28] and by Stehlik cum suis [18].

Summarizing, it may be stated here that all methods reported for nicotine and/or cotinine assay in biological fluids known to us are afflicted with some kind of drawback. It is not amazing that Davis in his article [28] noticed that there had been no reports specifically mentioning automated sample injection in the determination of nicotine and cotinine. The majority of the work-up procedures is so laborious that the number of extracts that can be produced in the course of one working day is hardly worth while to involve an automatic injector in their chromatographical analysis.

This paper for the first time describes an assay procedure requiring only a single extraction step which without any modifications can be applied to extract nicotine and cotinine rapidly, simultaneously and nearly quantitatively from plasma, saliva and urine. The assay furthermore resolves nicotine and cotinine from a single injection within 10 minutes, and is sensitive enough to be applied to the urine of passive smokers. 45 samples can be extracted and run chromatographically by a skilled analyst in a single 8 h working day.
These characteristics are accomplished by combining solid phase extraction work-up with high resolution gas chromatography, oven temperature programming, splitless injection and nitrogen-phosphorous detection.

2.3 MATERIALS AND METHODS

Glassware All glassware for sample storage and for the extraction procedure was cleaned by leaving it overnight in concentrated nitrous acid, washing it consecutively with hot tap water and ethanol, and drying it in an oven at 60°. The glassware was subsequently silanized by exposing it to a 4% dichloro dimethyl silane solution in toluene, which was left in it overnight or for at least 3 h. It was then washed with ethanol and briefly dried in an oven at 60°. When not in use, it was stored in dust-free and air-tight boxes.

Laboratory and personnel An interdiction to smoke in the analytical laboratory was issued one year before the first authentic samples were analyzed. The analyst and the blood sampler were chosen non-smokers.

Chemicals and solvents (-)-Nicotine was purchased from Merck, Darmstadt, West-Germany, (-)-cotinine from Sigma, Saint Louis, USA, nikethamide from Janssen Chimica, Beerse, Belgium, β-nicotyrine was a kind gift from Dr Th Darby of the Department of Pharmacology, University of South Carolina (Columbia, S C, U S A). These four compounds, whose structural formulas are shown in figure 1, were purified by double distillation under nitrogen before use. A purity of ≥ 99% was thus achieved, as revealed by gas chromatography and mass spectroscopy. The compounds were stored dark at -20° in desiccators under reduced (nitrogen) pressure to prevent oxidation, degradation and contamination.

Every bottle of the following solvents was checked individually on the presence of nicotine contamination by gas chromatographical analysis described herein: ethanol (p a grade, Merck), methanol (p a grade, Merck), propyl acetate (p a grade, Janssen Chimica) and toluene (technical quality, Boom, Meppel, The Netherlands). A 0.05 M solution of disodium tetraborate (Janssen Chimica) in tap water was buffered to pH = 9.0 by adding a few drops of concentrated nitrous acid (Merck), an aliquot of 10 ml of the buffer was checked on the presence of nicotine by solid phase extraction followed by gas-
chromatographical analysis (see 'Procedure') The solvents and the buffer solution were rejected if more than 1 ng of nicotine was found in the 10 ml aliquot

Instrumentation The gas-chromatographical analyses were performed on a Hewlett Packard model 5890 instrument, equipped with a split/splitless liner, a splitless insert (79 x 2 mm ID), a HP fused silica capillary column (12.5 m x 0.20 mm ID) wall-coated with a dimethyl silicone phase (phase ratio = 150) and a nitrogen-sensitive detector containing a rubidium bromide crystal detector gas flow rates were 3.0, 75 and 30 ml/min for hydrogen, air and make-up helium, respectively. The linear velocity of the helium carrier gas through the column was 42 cm/sec, achieved with a column head pressure of 1.0 Bar. The injection port and the detector were operated at temperature of 200° and 300°, respectively.

Sharp peaks with minimal tailing were obtained by splitless injection of propyl acetate sample extracts at an oven temperature of 60°, followed after 30 sec by activation of the septum purge (1.5 ml/min) and a rise of the oven temperature at 70°/min to 140°, then at 5°/min to 155°, and finally at a rate of 30°/min to 200°. The latter temperature was maintained 45 sec in case of plasma or saliva analysis or 90 sec in case of urine analysis. Subsequently, the oven was allowed to cool down back to 60°.
Plotting of the chromatograms was carried out by a flow chart recorder (model BD-8, Kipp & Zonen, Worms, The Netherlands). Electronic peak integration was performed by a Hewlett Packard 21 MX Lab Auto System. A Texas Instruments data terminal (model 700 ASR 'silent') read the integration options and printed out the reports.

Procedure. After thawing, the samples were vortex-mixed and centrifuged for 5 min at 1000 g. A 1 ml plasma sample or 100 μl urine sample was pipetted into a test tube followed by aliquots of the appropriate internal standard solutions (see: 'Calibration') and 1 ml of borax buffer (pH = 9). The contents of the tube were mixed well and then were passed under suction (flow rate about 0.5 ml/min) through a C2 solid phase extraction (SPE) column containing 100 mg of ethyl-silica sorbent (JT Baker, Phillipsburgh, USA). The SPE-column had been preconditioned by a 2 ml methanol wash and a 2 ml borax buffer wash. A Baker 10TH-SPE® vacuum manifold allowed 10 samples to be extracted at one time.

After passage of the sample, the SPE-column was washed twice with a 2 ml portion of distilled water and once with 100 μl of an aqueous methanol (80%) solution. Then it was dried thoroughly under air suction for 5 min. Nicotine, cotinine and their ISTDs were desorbed under gravity from the SPE-column with 500 μl of methanol. The eluates were caught in conical 1 ml vials. The methanol was evaporated at 45°C under a gentle stream of dry nitrogen in 30 min. It was exchanged with propyl acetate by addition of 20 μl at the start of the evaporation, which was terminated at a residual volume of 10 μl. A 0.75 μl aliquot was injected into the gas chromatograph.

Calibration. Blank plasma was obtained from nonsmokers working in a smoke-free environment. Blank urine was obtained from a 5 years old boy whose parents were both non-smokers. No cotinine could be detected in these fluid pools; nicotine levels were less than 0.3 ng/ml in both plasma and urine. Nicotine levels found in dog or goat plasmas or even in tap water were similar in magnitude and ranged from 0.1 to 0.5 ng/ml. Probably these specimens do not contain nicotine but are contaminated with it during the assay procedure.

Stock and working standard solutions of nicotine, cotinine, and their respective ISTDs β-nicotyrine and nikethamide were prepared in borax buffer (pH = 9) and stored at 4°C in the dark. They were refreshed every 5 working days.
10 plasma or urine calibration standards were prepared. Blank plasma (1 ml) was spiked with 50 ng of β-nicotyrine and 250 ng of nikethamide as IS (Internal Standard) with 0 (0) - 5 (40) - 10 (80) - 15 (120) - 20 (160) - 25 (200) - 30 (250) - 40 (300) - 50 (350) - 60 (400) ng of nicotine and cotinine (spiked amounts of cotinine given in brackets) Blank urine (0.1 ml) was spiked with 100 ng of both IS (Internal Standards) and with equal amounts 0 - 10 - 20 - 30 - 40 - 50 - 60 - 75 - 87 - 100 ng of nicotine and cotinine. The standard samples were taken through the extraction and chromatography procedure in the normal way.

Calibration points were generated by plotting the amounts of nicotine and cotinine in the standard sample versus the peak area ratio of nicotine to β-nicotyrine and cotinine to nikethamide, respectively. Nicotine and cotinine concentrations in authentic samples were calculated according to the linear least squares regression line through the nicotine or cotinine calibration points.

Extraction and overall recovery. Each sample of three groups of ten blank plasma samples (1 ml) was spiked with 50 ng of β-nicotyrine and 250 ng of nikethamide. Group I was also spiked with 25 ng of nicotine and 250 ng of cotinine. After the three groups of samples had been taken through the extraction procedure, the ten samples of group II were still spiked with 25 ng of nicotine and 250 ng of cotinine as methanolic solutions. The three groups of samples were then taken through the concentration procedure. The samples of group III then were spiked with 25 ng of nicotine and 250 ng of cotinine as propyl acetatic solutions.

The mean peak area ratio of nicotine / β-nicotyrine and of cotinine / nikethamide were determined in each of the three groups of samples. The extraction recoveries of nicotine and cotinine were calculated by dividing the mean peak area ratios in group I by the corresponding ones in group II. Similarly, the mean ratios in group I divided by those in group III yielded the total recoveries of nicotine and cotinine. Total recoveries were determined both at a residual evaporation volume of 50 µl and at the usual volume of 10 µl, by injection of an aliquot of the group I samples at both these residual volumes and subsequent division of the resulting mean peak area ratios by the corresponding figures in group III.

Extraction and overall recovery of nicotine and cotinine in 0.1 ml urine samples were determined using the same strategy, spiking the samples with 100 ng of each of the standards and adding 50 ng of both nicotine and cotinine to the sample groups at the appropriate stages of work-up.
The extraction and evaporation recoveries of the standards β-nicotyrine and nikethamide from plasma and urine samples were estimated by inversion of the strategy nicotine and cotinine were used as ISTDs for β-nicotyrine and nikethamide, respectively, and the latter two compounds were added at the various stages of work-up.

2.4 RESULTS AND DISCUSSION

Contamination Extraneous nicotine present in the atmosphere, in solvents and reagents, and on the surface of glassware, apparatus and the skin of the venipuncturist or analyst may be incorporated into the biological samples and thus falsely elevate nicotine levels in these samples. This elevation has been minimized by precautions like the issue of a smoke interdiction in the analytical laboratory, the appointment of a non-smoking blood sampler and analyst, the meticulous cleaning of all glassware followed by the application of an anti-adsorptive dimethylsilyl coating on its inner surfaces, and the avoidance of all unnecessary contact between the sample and plastic, metal, and uncoated glass surfaces. Furthermore, the total work-up of the samples was performed in a fume-hood, and all reagents and solvents were checked for their purity.

It thus has been possible to reduce the nicotine concentrations of 'blank' plasma and urine samples (and the y-intercepts of nicotine regression lines) to a value below 0.5 ng/ml (see 'Calibration'). For urine samples contamination with extraneous nicotine is less of a problem though, since at least in 'active' smokers the level of contamination is only a minute fraction of the true nicotine level of the samples. Since tobacco smoke contains only traces of cotinine whereas biological fluids of smokers contain relatively much of it, no contamination problems whatsoever exist in cotinine analysis.

Extraction and overall recovery Extraction using ethyl-silica ('C2') solid phase extraction columns under the conditions described resulted in an almost quantitative extraction of both nicotine, cotinine and their ISTDs from human plasma and urine (see Table I).

The extraction recovery of all compounds from urine appears to be marginally greater than that from plasma, which could be due to a slight extent of plasma protein binding of the compounds. In the range of concentra-
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Compound</th>
<th>Extraction recovery (%)</th>
<th>Overall @ recovery (%)</th>
<th>Overall @@ recovery (%)</th>
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<td>nicotine</td>
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<td>93 ± 5</td>
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<td>ß-nicotyrine</td>
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<td>94 ± 5</td>
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</tr>
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<td>nikethamide</td>
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<td>nikethamide</td>
<td>100 ± 3</td>
<td>99 ± 3</td>
<td>98 ± 3</td>
</tr>
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</table>

@ for a residual evaporation volume of 50 μl
@@ for a residual evaporation volume of 10 μl

Tions normally encountered in smokers' plasmas, and at pH = 7.4, nicotine is bound to plasma proteins for 4.9 ± 8.0% [35], whereas cotinine exhibits a bound fraction of 6.0 ± 3.5% [36]. We have not been able to trace the protein-bound fractions of ß-nicotyrine and nikethamide in the literature.

The plasma extraction recoveries of nicotine and cotinine are slightly greater than their free fractions in plasma. The difference might be explained by assuming that a slightly greater fraction of both amines is free at the pH of extraction (pH = 9) than at the physiological plasma pH (= 7.4). Alternatively, part of the protein-bound nicotine and cotinine molecules may move from the binding sites on the proteins to the competing sites on the extraction sorbent during passage of the plasma proteins through the SPE-column [37].

During the concentration step following extraction, approximately 20% of nicotine and its ISTD occur are lost, whereas the recoveries of the less volatile cotinine and its ISTD are hardly affected by the concentration procedure (Table I). Nicotine and ß-nicotyrine are lost mainly in the tail of the concentration step, from 50 μl to 10 μl. Nevertheless we decided to maintain 10 μl as a residual volume in the procedure because the factor 5 in volume reduction achieved in the tail of the concentration step is accompanied by a sensitivity gain that easily outweighs the 20% evaporation losses of nicotine and ß-nicotyrine.
Despite the incomplete recovery, the accuracy data for nicotine in the proposed method are beneficial by the grace of its equally volatile ISTD, which is recovered to practically the same, incomplete, extent (Table I). The importance of similarity in physico-chemical properties (volatility) between a compound and its ISTD thus is demonstrated, as is the necessity to employ dual internal standards for nicotine and cotinine. Like nicotine, β-nicotyrine is a tobacco alkaloid. However, its abundance in tobacco and tobacco smoke is infinitesimal, justifying its use as an ISTD in this assay method. We have not been able to detect smoke-derived β-nicotyrine in human plasma or urine so far. Trace quantities (= 1 μg) were found in cigarette filters.

Gas chromatography and sensitivity The specificity and the sensitivity of the nitrogen-phosphorous detector for compounds containing atoms of these elements is very suitable for an accurate estimation of even small concentrations of nicotine and cotinine in biological fluids. The detection limit at a signal to noise ratio of 5 corresponds with a splitlessly injected quantity of about 5 pg of nicotine and 15 pg of cotinine, which quantities in turn correspond to minimum detectable amounts of ≤ 0.1 ng of nicotine and 0.2 ng of cotinine per ml of plasma or per 0.1 ml of urine.

Considering that the simple single-step extraction procedure described herein provides a limited degree of sample clean-up, the gas chromatographical analysis following it should have a high resolving power. Excellent resolution was obtained by using a short (12.5 m) dimethyl silicone-coated capillary column combined with splitless injection under well-chosen values of critical parameters like inner insert volume (0.25 ml), carrier gas flow (0.8 ml/min) and purge activation time (30 s), an injection port temperature (200°) high enough to vaporize injected analytes and standards, but low enough to prevent their decomposition and prohibit the vaporization of compounds which could interfere in the chromatograms.

Figure 2 shows some representative chromatograms of plasma and urine extracts. The peaks are symmetrical and well-resolved, widths at half-height varying from 1 sec for nicotine to 3 sec for cotinine. The retention times are 30 - 38 - 45 - 57 min for nicotine, the ISTDs β-nicotyrine and nikethamide, and cotinine, respectively. Caffeine is unintentionally detected at 65 min. The chromatographical runtime including the cooling time of the oven (200° + 60°) comes to 95 min for a plasma extract and 10 min for a urine extract. Though no examples are shown, chromatograms with a similar appearance are obtained for the clear supernatant resulting from centrifugation (5 min, 1000 g) of human saliva.
FIGURE 2 Chromatograms of (a) a plasma extract from a casual smoker after 24 hours of smoking abstinence, (b) idem, after smoking 14 cigarettes in 8 hours, (c) a morning urine extract from a 7 year old boy exposed to the smoke of 36 cigarettes smoked by his parents during the preceding day, and (d) a urine extract from a package-a-day smoker. Peaks 1 = nicotine, 2 = β-nicotyrine (ISTD₁), 3 = nikethamide (ISTD₂), 4 = cotinine, 5 = caffeine. Concentrations of nicotine / cotinine (a) 0.5 / 120 (b) 37 / 390 (c) 21 / 18 (d) 250 / 265 ng/ml. Attenuation (a & b) (a) and (b) 250, (c) 125, (d) 1000 (×10⁻¹²)

After injection of about 100 extracts, deterioration of chromatographic separation occurred as a result of the formation of carbonaceous deposits on the interior surface of the insert and in the column head. In such an event, the polluted insert was replaced by a spare one, cleaned in chromic acid, rinsed in various solvents, and deactivated (see 'Glassware'). In addition, 20 cm of the column was broken off at the injector end and the column was reinserted into the injection port. The column length of 12.5 m reported here was in fact the mean of an initial length of 15 m and a terminal length of 10 m. At this minimal length, the nicotine peak sometimes showed overlap with nearby endogenous peaks.
FIGURE 3 Concentration profiles of nicotine (o) and cotinine (*) in the plasma of a steady smoker during 24 h. Each bottom triangle denotes the (time of) smoking of a single cigarette.

The longevity of the collector bead was determined by the deposition rate of silicone particles originating from the column ('silicone bleed'). On the average a collector being in almost daily use lasted 6 months, which is equal to the maximum lifetime stated by the manufacturer.

Nicotine and cotinine plasma concentrations were determined over a 24 h period in a moderate smoker of a non-ventilated filter-tipped cigarette brand (13 mg of tar, 1.0 mg of nicotine) according to the proposed analytical method. Before analysis, the samples had been stored for 2 months at -20°C. The concentration profiles are shown in figure 3.

Accuracy Detector response is linear over the entire range of nicotine and cotinine concentrations added to the calibration standard samples. Correlation coefficients of the calibration curves are between 0.990 and 0.999 (average coefficient: 0.994). Calibration curves from day to day have reproducible slopes that approximately equal the reciprocal of the spiked amount of ISTD, expressed in nMoles. This is an indication that the detector response to
<table>
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<th>Spiked conc. ng/ml</th>
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<th>Standard deviation ng/ml</th>
<th>CV % (n=5)</th>
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nicotine and cotinine is comparable with the response to their respective ISTDs. The mean intercept of successive cotinine regression lines is not significantly different from zero, that of successive nicotine regression lines can be kept as low as a few tenth of ng of nicotine per ml of plasma or per 0.1 ml of urine if the given procedure for the cleaning and silanizing of glassware is applied uncurtailed. To reduce carry-over owing to the adsorption of nicotine and cotinine in the syringe, the latter was rinsed in vials containing 10 ml of 2-propanol and 1.5 ml of concentrated ammonium hydroxide between injections.
Reproducibility characteristics were acquired by spiking blank 1 ml plasma samples and 0.1 ml urine samples with the usual amounts of ISTDs (see 'Calibration') and with nicotine and cotinine to give samples of different concentrations within the range 1 - 60 ng of nicotine and 5 - 400 ng of cotinine per ml of plasma, and within 50 - 1000 ng of both nicotine and cotinine per ml of urine. Five samples at each concentration were prepared and analyzed. At each concentration the mean and the standard deviation of the observed concentration and the coefficient of variation (CV) was calculated. The values are displayed in Table II. The results show that plasma concentrations of nicotine ≥ 2 - 5 ng/ml, plasma levels of cotinine ≥ 5 - 25 ng/ml, and urinary concentrations of both alkaloids exceeding 50 - 100 ng/ml are measurable with a CV of ≤ 10% or less. These concentrations nicely cover the range of nicotine and cotinine levels occurring in moderate smokers (e.g., see figure 3).

2.5 CONCLUSIONS

For the first time an assay procedure is described for the simultaneous analysis of nicotine and cotinine which 1) requires only one extraction step in which both alkaloids are extracted rapidly and nearly quantitatively, which 2) is applicable to plasma and urine without modifications, and which 3) separates both alkaloids from each other and from endogenous plasma and urine constituents in a single and short gas-chromatographical run.

These qualifications were achieved by combining solid phase extraction, high resolution capillary gas chromatography, splitless injection, and nitrogen-specific detection. The rigorous exclusion of extraneous nicotine and the structural analogy and resultant analogy in physico-chemical characteristics existing between nicotine, cotinine and their respective internal standards also contribute to the reliability of the method.

In our laboratory the method is being used routinely to determine the pharmacokinetics of nicotine and cotinine and to estimate the nicotine intake of 'active' and 'passive' smokers. More than 1000 samples have been analyzed for nicotine and cotinine so far by the procedure described.
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Chapter 3

ESTIMATION OF THE NICOTINE CONTENT OF CIGARETTE FILTERS AND GLASS FIBRE ('CAMBRIDGE') FILTERS

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3.1 INTRODUCTION

A sample preparation procedure composed of crushing, ultrasonic vibration, and filtration was combined with a shortened version of the GC-NSD procedure presented in the previous chapter to quantify nicotine in filters of man-smoked and machine-smoked cigarettes, and in glass fibre ('Cambridge') filters used for trapping mainstream smoke aerosols during machine smoking.

The mainstream smoke is that part of the smoke that is generated during a puff and is subsequently drawn through the cigarette and its filter by suction applied to this filter by the smoker or the smoking machine. Part of the aerosols containing water, dry smoke condensate ('tar') and nicotine (which occurs almost completely associated with aerosols in the (slightly acid) cigarette smoke) is deposited in the cigarette filter. In human smoking, the complementary part of these substances is delivered to the smoker's mouth, together with the gaseous phase of the mainstream smoke which is virtually unaffected by most commercial cigarette filters. In machine smoking, the mainstream smoke may be deprived of its remaining aerosols by filtering it through a 'Cambridge' filter, trapping more than 99% of the particles by weight. After filtration, the particle phase and the remaining gaseous phase of the smoke may be separately analyzed for their substance composition.

A quantitative analysis procedure for nicotine in cigarette filters will now be described. In addition, a slight modification of the assay, rendering it applicable to analyze 'Cambridge' filters for their content of the alkaloid, is given in a note at the end.
Materials  A household blender/mixer (Moulinex model Moulinette 643 S, France) was purchased locally. Cellulose acetate filters (diameter 2.5 cm, pore diameter 0.45 μm) were obtained from Schleicher & Schull, Dassel, West Germany. Single-use polypropylene 2 ml-syringes ('Monoject') were from Ballymoney, Northern Ireland. The ultrasonic bath was a Sonicator model SC-120T (Sonicator Instrument Corp, New York). The origin of the chemicals and the other materials is specified in the section 'Materials and Methods' of Chapter 2.

Standard solutions  Solutions of nicotine (30 mg/l) and of β-nicotyrine (250 mg/l) were made up in propyl acetate. If stored at 4°C protected from light, their stability sufficed to be used for at least two weeks.

Procedure  To ensure the highest accuracy, the cigarette butts to be analyzed were stored in air-tight bottles in the dark at room temperature by the subjects themselves and later in the laboratory. The 10 butts selected for analysis from the daily pools collected by the subjects had a tuft of unburnt tobacco occluding the distal end of the filter, and they were free of lipstick stains as a potential source of substances interfering with the gas-chromatographic analysis [1]. Furthermore, the mean length of the selected butts coincided with the mean length of the butts rejected for analysis. Butts originating from the 16-day collection period of each subject were processed within-day according to the following directives.

After removal of ashes and residual unburnt tobacco, the filters of the 10 selected butts were crushed in a household blender for 30 sec. The resulting mount of filter fibres was quantitatively transferred into an Erlenmeyer flask containing 100 ml of methanol. The flask was stoppered and its suspended contents were subjected to ultrasonic vibration for 30 min. Part (5-7 ml) of the suspension was filtrated through a cellulose acetate filter (pore diameter 0.45 μm). A 25 μl aliquot of filtrate was pipetted into a 1 ml vial, to which an equal volume of the β-nicotyrine standard solution was added as an external standard. The contents of the vial were diluted with 600 μl of propyl acetate and thoroughly mixed. Part (0.4-0.6 μl) of the mixture was injected under the conditions described in Chapter 2 ('Instrumentation'). However, the GC oven temperature program was cut off at 152°C after elution of the compound appearing latest, which is the ESTD ($R_t = 3.8$ min). As a blank control sample, ten filters of unsmoked cigarettes of the same brand were processed analogously.
Calibration. Seven standard samples were prepared by transferring 0-10 - 20 - 30 - 40 - 50 - 60 μl volumes of the nicotine standard solution into 1 ml-vials. Addition of the ESTD and propyl acetate to these standard samples and their gas-chromatographic analysis were carried out as specified in the previous paragraph. A calibration curve was constructed by plotting the nicotine/ESTD peak area ratio (Y) versus the amount of nicotine present in the standard sample (X). A handcalculator program calculated the slope 'a' and the intercept 'b' of the regression line (Y = aX + b) of the calibration curve.

The absolute amount of nicotine ($F_{uncorr}$) in 10 filters giving rise to a peak area ratio 'y' can be shown by trivial calculation to average:

$$F_{uncorr} = \frac{400 \ (y - b)}{a} \ (\mu g/\text{filter})$$

The nicotine amount in unsmoked cigarette filters ($F_{unsm}$) is calculated the same way. The gross nicotine retention in filters of smoked cigarettes ($F_{uncorr}$) is corrected for the amount of nicotine present in unsmoked filters to yield the average amount of nicotine in the filters, deposited there by the act of smoking ($F$):

$$F = F_{uncorr} - F_{unsm} \ (\mu g/\text{filter})$$

Throughout this dissertation, amounts of nicotine stated to be present in cigarette filters are corrected for nicotine present in these filters before smoking.

Nicotine filter retention as a function of storage time. To obtain an indication of the rate at which nicotine is lost in filter butts during storage, we requested a smoker consuming 25 - 30 cigarettes/day to collect her smoked-out butts. Butts were gathered during a period of 16 consecutive days; daily saved butts were pooled in 50-ml brown polypropylene pots with a screw-cap and stored in the way indicated in the beginning of this section.

At the 17th day from the 16 portions of butts, two groups of 10 butts were selected having estimated equal average length, a tuft of unburnt tobacco at the distal end, and no sign of lipstick stains. The first group of 10 butts was analyzed on this very 17th day; the other group of 10 butts was carefully placed back into the screw-capped pots and was not analyzed until 14 days later (at the 31th day). The results of the analyses were compared.
3.3 RESULTS AND DISCUSSION

Chromatography Figure 1 (a and b) displays chromatograms of 20 cigarette filters, selected from a total of 31 butts pooled by the volunteer at the 7th day of his collection period. One group of 10 butts was analyzed for its nicotine content when 10 days old, the other group of again 10 butts was analyzed 14 days later. The chromatograms do not differ, except for the ratio of nicotine to ESTD which is slightly smaller for the older group of butts. Figure 1 c shows that the amount of nicotine present in filters of unsmoked cigarettes of the same brand is relatively small, though not negligible.

The chromatograms have a clean appearance. A minor unidentified peak is visible between the peaks of nicotine and the ESTD. The unknown peak constitutes a contaminant of the ESTD-solution, its identity probably being N-methyl myosmine (suggested by mass spectrometry).

The retention times are 3.0 min for nicotine and 3.8 min for the ESTD β-nicotyrine. The peaks are very well resolved, peak widths at half-height varying from 1 sec (nicotine) to 2 sec (ESTD).

Filters of smoked cigarettes of the brands investigated in the course of this research were found to contain 0.4 - 3.0 mg of nicotine, depending on brand and smoking technique, whereas unsmoked filters typically contained 20 - 40 μg of it. The latter amount probably is deposited in the filter by inward diffusion of nicotine migrated from the tobacco adjacent to the filter.

Although the ESTD β-nicotyrine is a tobacco alkaloid itself, it occurs only in trace quantities of a few micrograms or even less in filters of smoked cigarettes. Therefore its use as an external standard in this analytical method is validated.

Accuracy Correlation coefficients of regression lines varied from 0.990 to 0.999 (average 0.996). The total recovery of nicotine in this method solely depends on the efficacy of detachment of nicotine from the crushed filter fibres in methanolic milieu, which probably is complete. The working-up procedure does not include extraction or evaporation steps in which detectable losses could take place. Another factor that might give rise to nicotine losses is the transfer step of the crushed filter fibres into the flask containing 100 ml of methanol. However, an experienced analyst will recover more than 99% during transfer.
FIGURE 1 Chromatograms of cigarette filters (a) a set of 10 smoked-out filters, collected by a smoker at the 7th day of a 16-day collection period, and analyzed when they were 10 days old, (b) another set of 10 filters, collected by the same smoker at the same day, analyzed when they were 24 days old, (c) 10 filters of unsmoked cigarettes of the same brand. Peaks 1 = nicotine, 2 = contaminant, 3 = ESTD (β-nicotyrine). The nicotine peak in chromatogram (b) is slightly lower than in (a), indicating a small nicotine loss having occurred between the 10th and the 24th day of storage (~4 3%, cf Table I).
non-reproducible amount of nicotine already present in filters of non-smoked cigarettes Therefore this strategy was rejected

Nevertheless, the CV may be 2.5% at most, 2.5% being the CV determined for the analysis of nicotine at high concentrations in biological fluids We feel that this estimate is realistic, because

- the recovery of nicotine is more reproducible and higher in filter analysis than in the analysis of biological fluids, and
- analytical methods in general tend to be more precise with the amount of material to be analyzed

The concentration of nicotine in the methanol suspension of crushed filters (typically 50 μg/ml) exceeds that in blood plasma (typically 20 ng/ml) by three orders of magnitude

Nicotine filter retention as a function of storage time As filter butts of smoked cigarettes age, they desiccate The 'desiccation' process in a broad sense applies both to the water content and to the nicotine content of the filter

Nicotine is lost from the filter through chemical degradation, evaporation ('sublimation'), and maybe photolytic decomposition The rate of the latter process may be effectively reduced to zero by storing the filters in the dark Evaporation is minimized by storing the filters in an air-tight flask at temperatures not higher than ambient Moreover, evaporation is retarded if the butt contains a residual tuft of tobacco sealing the distal end of the filter

The desiccation process in butts stored under the above-mentioned optimal conditions was investigated as a function of time as described under 'Materials and Methods' Figure 2 shows the results of the first analysis, whereas Table I compares the results of the first and the second analysis

The agreement of the results of the first and the second analysis is closer than 10%, in spite of desiccation and notwithstanding the fact that each portion of butts must be considered to be unique It may be concluded that 10 cigarettes are a good random check to determine the daily average nicotine butt content The nicotine content of individual cigarette butts must be suspected to vary considerably though, as does the average content from day to day (figure 2)

The rate at which nicotine disappears from these butts appears greatest in the first five days of butt storage, it amounts to some 6% (1.2% per day) of the amount present in the filters We associate this rapid loss with nicotine
FIGURE 2 Daily average of the nicotine content of 10 butts of filter cigarettes, smoked and collected by a smoker during 16 consecutive days. For each of the 16 days a representative check of 10 smoked filters was taken and collectively analyzed at the 17th day. The last bar shows the mean filter nicotine content (± SD) in the whole period. Note the large differences in nicotine content from day-to-day, which likely correspond to proportional differences in smoking technique.

Evaporation from superficial areas of the filters. In the weeks thereafter, nicotine is lost at a slower rate, presumably from deeper layers of the filters (0.3 - 0.4 % per day).

These figures are comparable to the 13 % average nicotine loss reported in reference [2] for 62 portions of 10 butts of ventilated filter cigarettes stored for a period of 14 days.

The mean age of the butts saved by our volunteers in the course of a pharmacokinetic investigation of the nicotine intake by human smokers came to 9 days. As a tentative conclusion on the basis of the available data (Table I), we expect a mean percentage of approximately 7½ % of nicotine to be lost from the butts during storage.

The nicotine intake per cigarette at mouth-level (D) and at blood-level (D*exp.), and the nicotine intake rate (Ḋ) given elsewhere in this thesis were
### TABLE I  LOSS PERCENTAGES OF NICOTINE IN CIGARETTE FILTERS AS A FUNCTION OF STORAGE TIME

Butts collected by the smoker at the \((17-\xi)_{th}\) day \((\xi = 1, 2, 16)\) of the 16-day collection period were analyzed at an age of \(1\) days, and \(1+14\) days, if assigned to 'short' and 'prolonged' storage groups, respectively.

<table>
<thead>
<tr>
<th>Butt age(^1)</th>
<th>Butt age(^2)</th>
<th>Loss(^3)</th>
<th>Butt age(^1)</th>
<th>Butt age(^2)</th>
<th>Loss(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>days</td>
<td>%</td>
<td>days</td>
<td>days</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>-9 2</td>
<td>9</td>
<td>23</td>
<td>+1 6</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>-7 8</td>
<td>10</td>
<td>24</td>
<td>-4 3</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>-6 5</td>
<td>11</td>
<td>25</td>
<td>-4 3</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>-5 4</td>
<td>12</td>
<td>26</td>
<td>-0 2</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>-4 0</td>
<td>13</td>
<td>27</td>
<td>+2 9</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>-3 0</td>
<td>14</td>
<td>28</td>
<td>+3 0</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>-3 5</td>
<td>15</td>
<td>29</td>
<td>-4 1</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>-2 1</td>
<td>16</td>
<td>30</td>
<td>+1.0</td>
</tr>
</tbody>
</table>

\(^1\) Age of short-stored group of butts at analysis (analysis result \(F_1\) mg)

\(^2\) Age of longer-stored group of butts at analysis (analysis result \(F_2\) mg)

\(^3\) Calculated as \(\{(F_2 - F_1)/F_1\} \cdot 100\%\)

not corrected for this loss, because we felt that the body of available data on nicotine losses from the stored butts was too limited. The listed values of the former-mentioned intake quantities therefore are slight underestimations. Unfortunately, a more profound investigation of the rate of the nicotine loss process was not feasible because of lack of time.

### 3.4 NOTE ON THE ANALYSIS OF GLASS FIBRE FILTERS

The analytical procedure for estimating nicotine amounts trapped on 'Cambridge' glass fibre filters is essentially as described in the Dutch standard NEN 7382 [3] and is comparable to the one used to estimate the amounts deposited in cigarette filters.

The particulate matter in the mainstream smoke of 10 cigarettes smoked on a machine is deposited on a 'Cambridge' filter. The glass fibre filter is removed from the holder and immersed in 100 ml of methanol. Particulate matter deposited on the inner walls of the holder is wiped off using a pair of tweez-
ers with half a clean, folded 'Cambridge' filter, which is then also immersed in the methanol. The Erlenmeyer flask containing the filters and the methanol is shaken for 15 min. It is then stored overnight. An aliquot sample of the supernatant liquid is used for analysis as described in § 3.2 after filtration. A blank is prepared by the same procedure using one and a half clean 'Cambridge' filter.

3.5 CONCLUSIONS

The method presented here for the estimation of the absolute retention of nicotine in filters of smoked cigarettes is rapid and precise. It is well-documented with respect to the way in which filter-tipped butts should be stored in order to minimize nicotine losses from them pending analysis. An indication of the relative nicotine loss rate from the filter butts as a result of storage for a period up to one month has been given. Analytical results are only slightly affected by a storage time of 9 days.

3.6 REFERENCES

CAPILLARY GASCHROMATOGRAPHIC - MASS SPECTROMETRIC - SELECTIVE ION MONITORING METHOD FOR MEASURING RELATIVE LEVELS OF COTININE AND ORALLY ADMINISTERED COTININE-D2 IN SMOKERS' BLOOD PLASMAS

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4.1 INTRODUCTION

While designing the investigation of nicotine intake of human cigarette smokers, the need arose for an analytical method that would enable us to separately detect nicotine's principal metabolite cotinine and a deuterium-labelled cotinine analogue 4',4'-dideutero cotinine (cotinine-D2) in smokers' plasmas. We synthesized the latter cotinine analogue with the intention to administer it perorally to our experimental smokers in order to reveal a basic pharmacokinetic parameter (total clearance), which is identical for cotinine-D2 ('native' cotinine) and for cotinine-D2.

In this thesis a capillary column gas chromatographic method with nitrogen-sensitive detection is described for the analysis of nicotine and cotinine in biological fluids. By its very nature, a nitrogen sensitive detector cannot distinguish between a molecule and the same molecule provided with an isotopic label. To attain our goal, we replaced nitrogen-sensitive detection by mass spectrometry (MS) with the facility of selected ion monitoring (SIM). As the method is not aimed at nicotine analysis, it became feasible.

Present address Turmac Tobacco Co, P O Box 12, 6900 AA Zevenaar, The Netherlands
to speed up the GC oven temperature program as well.

The resulting method does not employ an internal or external standard. Hence relative concentrations of cotinine and cotinine-D2 are measured. Stated differently, the concentration ratio of cotinine and cotinine-D2 is determined rather than the absolute concentrations of these compounds. The latter are calculated by combining the relative concentrations obtained by the presently described GC-MS-SIM method with the total cotinine concentration (the sum of the concentrations of cotinine and cotinine-D2) as analyzed by GC-NSD. This way of combining analytical data obtained by GC-NSD and GC-MS-SIM departs from the virtually identical physico-chemical properties of cotinine-H2 and its deuterated counterpart, particularly qua ionizability, and pattern and efficiency of fragmentation. We now proceed to describe the GC-MS-SIM method.

4.2 MATERIALS AND METHODS

Glassware, laboratory and personnel The procedure followed to obtain clean silanized glassware free of cotinine was outlined before. Again, a non-smoking analyst (R.A.) performed the analyses in a smoke-free laboratory.

Chemicals and solvents The reader is referred to the homonymous section in chapter 2 for the origin of the bulk of the chemicals and solvents. 4',4'-dideutero cotinine was synthesized in eight steps departing from nicotinic acid and pyrrolidone according to a procedure outlined in Appendix I of this thesis. It was found to be ≥95% chemically pure by spectrometrical techniques (UV, IR, 1H-NMR) solely, or combined with chromatography (reversed-phase HPLC with UV-detection at 254 and 280 nm, GC-NSD, GC-MS).

Instrumentation. The analyses were performed on a Hewlett Packard Model 5790 gas chromatograph equipped with a split/splitless capillary injector, a splitless liner and a fused silica capillary column (17.5 m x 0.20 mm ID) wall-coated with a dimethyl silicone liquid phase (phase ratio = 150, Hewlett Packard) The gas chromatograph was coupled to a Hewlett Packard Model 5970 quadrupole Mass Selective Detector by insertion of the capillary column into the GC-MS interface at a distance of less than 1 mm from the ion source block. The gas chromatograph and the mass selective detector were connected to a HP 9816 terminal/data system with implemented peak integration software.
The GC-MS system was operated at the following temperatures: injection port: 250°, interface and ion source block: 270°, oven temperature: 75° during injection, at 30 sec after injection followed by a semi-ballistic raise to 180°, a further raise at a rate of 20°/min to 220°, and finally a raise at 64°/min to 250°, which oven temperature was held for 1.5 - 2.5 min in case of a plasma sample and for 2.5 - 4.5 min in case of a urine sample. The precise dwelling time at 250° depended on the occurrence of late-eluting endogenous peaks in the samples, this occurrence varying between subjects.

The mass spectrometer was operated with an electron ionization voltage of 70 V and an accelerating voltage of 1800 V. The source pressure varied from 4*10^{-5} to 6*10^{-5} Torr with the GC oven temperature. The carrier gas pressure drop amounted to 1.0 Bar, resulting in a carrier gas (helium) flow rate of 0.5 ml/min. The make-up gas (helium) flow rate was 25 ml/min and the purge flow rate was 2 ml/min, except in the 30 sec during and following injection of a sample. 3 μl samples were injected in the splitless mode.

Prior to the beginning of a series of measurements, the ion source and the mass analyzer voltage settings were optimized to monitor the precise weight of the cotinine and cotinine-D2 base peaks (m/z = 98 and 100, respectively). For this purpose a volume of 3 μl propyl acetate containing about 50 ng of both cotinine and cotinine-D2 was injected into the GC-MS system. Six mass channels of 0.05 u difference around the theoretical mass value were monitored. Peak integration in each channel yielded the channel showing the highest intensity. The mass of the most intense channel in the 98 u-range was taken as the cotinine base peak, that in the 100-u range as the cotinine-D2 base peak.

Solid Phase Extraction Procedure. The extraction procedure is identical to the procedure described in chapter 2 for the GC-NSD method. In practice, extracts of each sample containing both cotinine-H2 and deuterated cotinine were divided in two. One aliquot was chromatographed with GC-NSD to determine its nicotine and (total) cotinine concentration, whilst the other aliquot was injected into the GC-MS combination to determine the ratio of the cotinine-H2 and cotinine-D2 concentrations.

Total recovery and accuracy. The total recovery of cotinine-H2 in the above-mentioned extraction procedure is listed for various cotinine concentrations in Table I of chapter 2. On the basis of the structure identity existing between the two cotinines and the ensuing virtually identical physico-chemical
characteristics of the two compounds, their recovery data must be expected to be identical as well.

The accuracy and reproducibility of the described method were not determined a priori. An indication of the reproducibility of the method in plasma was acquired afterwards by comparing analysis results of duplicate samples from our experimental volunteers. Be $f_D$ and $f_D'$ the measured molar fractions of cotinine-D2 in a particular plasma sample and its duplicate, respectively, then the relative deviation $\Delta_{rel}$ of the two measurements is defined as

$$
\Delta_{rel} = \left( \frac{|f_D - f_D'|}{f_D + f_D'} \right) \cdot 100\%
$$

$\Delta_{rel}$ was studied in three cotinine-D2 plasma concentration ranges [0, 50>, [50, 100> and [100, -> ng/ml. The concentration of nicotine-derived cotinine in our smoking subjects always was in the latter range, mostly having a value in between 200 and 350 ng/ml.

### RESULTS AND DISCUSSION

**Mass spectrometry** Mass spectra of cotinine-H2 and cotinine-D2 are presented in figure 1. The integer base peaks of cotinine-H2 and cotinine-D2 are 98 and 100, respectively, these fragment masses corresponding to the (monol- ionized) pyrrolidine moieties of the cotinine molecule. The mass mantissa as determined by the procedure described in the section 'Instrumentation' was found to be identical for both cotinine species at any time, as is to be expected from the mass difference between hydrogen and deuterium, which equals unity up to 0.01 u. The mantissa mostly amounted to 0.5, sometimes however to 0.0 or to 1.0.

**Chromatography** Typical plasma chromatograms of a steady smoking volunteer are shown in figure 2. The (coinciding) peak of cotinine-H2 and cotinine-D2 has a retention time of 3.2 - 3.3 min and a peak-width at half-height of about 10 sec under the conditions specified.

It may be inferred that most of the chromatogram run time is used to elute numerous endogenous peaks, which are present in both chromatograms of both selected ions (figure 2c/d), most notably in the mass 98 chromatogram (figure 2c). The time interval of cotinine elution (3.1 - 3.5 min), however, is not significantly occupied by endogenous peaks.
FIGURE 1. Mass spectra of (a) cotinine-H2 and (b) cotinine-D2. Base peaks correspond to the mass of the 5-membered (pyrrolidine) rings. Masses of the molecular ions are also clearly discernible. Structures of intact molecules and base peak fragments are shown.

The proposed GC-MS-SIM analytical method is reasonably rapid, the time interval between injections being 14 minutes. Due to the adverse heat capacity characteristics of the GC-oven, approximately half of the runtime is needed to cool the oven from 250°C down to 75°C.

An example of cotinine plasma profiles determined with the aid of the presently described assay is given in figure 3. Though no examples are shown, the reported method is also valid to determine the cotinine species composition existing in saliva and urine of smokers after an oral dose of cotinine-D2.
FIGURE 2 Chromatograms showing total abundance of the masses 98 and 100 in (a) blank human plasma and (b) in the plasma of a smoking volunteer obtained ± 1 h after ingestion of 5.3 mg of cotinine-D2. Separate abundances of mass 98 and mass 100 in the plasma of chromatogram (b) have been indicated in chromatograms (c) and (d), respectively.

Accuracy and sensitivity An indication of the accuracy of the present analytical method was obtained by comparison of analysis results of duplicate samples. The relative deviation $\Delta_{\text{rel}}$ of 98 duplicate measurements, spread over three cotinine-D2 concentration ranges, is shown in Table I. $\Delta_{\text{rel}}$ is seen to increase as the cotinine-D2 concentration decreases, cotinine-H2 meanwhile assuming a high steady-state value (200 - 350 ng/mL). In the lower part of the 0 - 50 ng/mL range the method is less accurate, $\Delta_{\text{rel}}$ probably being higher than 10%. The low accuracy at these low cotinine-D2 levels is due to the relatively low sensitivity of the mass spectrometer as a cotinine detector. To compensate for reduced accuracy, duplicate plasma samples taken 24 hours after cotinine-D2 administration (figure 3) often were run twice on the GC-MS.
FIGURE 3 Plasma concentration vs time profiles of (o) nicotine, (*) cotinine, and (x) cotinine-D2 in a steady smoker as determined over a 24 hrs period by GC-NSD and GC-MS-SIM. Bottom arrows denote the time of smoking of single cigarettes. A 53 mg dose of cotinine-D2 was administered to the smoker per os in a gelatine capsule at 9:00 a.m.

4.4 CONCLUSIONS

A gas chromatographic mass spectrometric method employing selective ion monitoring, preceded by solid phase extraction of the sample, has been developed to analyze the relative concentrations of nicotine-derived cotinine (cotinine-H2) and a deuterated cotinine species (cotinine-D2) given by mouth to the body fluids (mainly blood plasma) of smoking volunteers.

The relative deviation of duplicate analyses is less than 10% at cotinine concentrations greater than about 25 ng/ml. It shares its working-up procedure with the GC-NSD method described elsewhere in this dissertation, and was combined with the latter method to reveal the absolute concentrations of cotinine-H2 and cotinine-D2 in smokers' plasmas.
TABLE I RELATIVE DEVIATION OF D Duplicate Plasma Analyses of the Cotinine-D2 Molar Fraction

\[ \Delta_{rel} = \text{relative deviation of duplicate samples, } n = \text{number of plasma samples in the specified concentration range} \]

<table>
<thead>
<tr>
<th>Concentration range of cotinine-[D2] (ng/ml) (1)</th>
<th>0 - 50</th>
<th>50 - 100</th>
<th>(\geq 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta_{rel} ) (mean ± SD) (%) (2)</td>
<td>7.2 ± 2.6</td>
<td>3 ± 1.0</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>(n)</td>
<td>24</td>
<td>49</td>
<td>25</td>
</tr>
</tbody>
</table>

\(1\) Concentration range of cotinine-D2: The concentration of cotinine-[H2] exceeds 100 ng/ml.

\(2\) \(\Delta_{rel} = \left| \frac{f_D - f'_{D}}{f_D + f'_{D}} \right| \times 100\%\), where \(f_D\) and \(f'_{D}\) are duplicate measurements of the molar fractions of cotinine-D2 in each sample.

\(3\) \(n = 98\). The plasma samples were taken from 7 volunteers (smokers of cigarette brand F2) participating in experiments aimed at determining nicotine intake by human cigarette smokers.

ACKNOWLEDGEMENTS

We gratefully wish to acknowledge Dr. J. Lemmens and Prof. Dr. B. Zwanenburg of the Department of Organic Chemistry, Faculty of Natural Sciences and Mathematics, University of Nijmegen, The Netherlands, for development of the preparative synthesis route to 4',4'-dideutero cotinine described, and for carrying out the actual synthesis.

4.5 REFERENCE

Chapter 5

RAPID AND SENSITIVE GAS CHROMATOGRAPHIC DETERMINATION OF CAFFEINE IN BEVERAGES AND BIOLOGICAL FLUIDS

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and * Robert M.M. Maes

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5.1 SUMMARY

A gas chromatographic procedure has been developed for the determination of caffeine in plasma, saliva and xanthine beverages. Using a 75 cm column packed with OV-17 and nitrogen-phosphorus selective detection, a suitable limit of analysis (coefficient of variation (CV) = 10.2 %) of 50 ng/ml in plasma starting with a sample volume of 1 ml was obtained. Within-day CVs at caffeine concentrations of 0.1 - 0.5 - 2.0 - 7.5 - 15.0 µg/ml in plasma were 7.7 - 5.6 - 4.8 - 3.8 - 3.4 %, respectively. The limit of detection, defined as the injected quantity of caffeine giving rise to a signal to noise ratio of 2 is 40 pg caffeine, corresponding to a plasma concentration of 1 ng/ml.

The procedure involves addition of the internal standard 7-pentyl theophylline and alkaline extraction of the sample with dichloromethane. The described method rivals any gas-chromatographic analysis procedure published so far in rapidness and accuracy.

Plasma and saliva caffeine concentrations were determined in a healthy male volunteer swallowing 400 ml of coffee. The calculated pharmacokinetic parameters, assuming complete caffeine absorption from the G.I. tract, agree with previously published values.
Caffeine is an alkaloid occurring in plants distributed widely throughout the world. Its consumption by man, almost exclusively as beverages of various kinds, reaches back to the Paleolithic and has unflaggingly continued until the present day. The daily per-capita intake of caffeine in the United States is estimated to average above 200 mg in recent times, 90% of this amount resulting from drinking coffee [1]. It is expected that Western European figures on caffeine consumption are in close agreement with the American ones. So, on a molecular basis, caffeine after ethanol probably occupies the second highest position on the list of mass-consumed pharmacological agents in Western Society.

Undoubtedly, the popularity of caffeine beverages depends on its stimulant action on the central nervous system resulting in the well-known effects of elevating mood, decreasing fatigue and increasing performance. Although caffeine is a potential drug of abuse (for a review of caffeine abuse see Gilbert, [2]), cases of abuse are rare. Furthermore, caffeine has a low toxicity, is relatively inexpensive and, if withdrawn, its absence implements only a mild abstinence syndrome. Therefore caffeine consumption does not constitute a significant medical or social problem and, unlike that of e.g., alcohol or tobacco, never has been the subject of governmental prevention campaigns focussed on especially adolescents. Significantly, 'teetotalism' does not ban the teetotaller from enjoying coffee and caffeine.

Altogether, it looks much like the morning cup of coffee or tea will remain part of the Western dietary habit for a long time to come, an expectation that stresses the need for further investigations into the intake, the pharmacokinetics, metabolism and pharmacodynamics of caffeine.

As the initial stage of such an investigation, we developed several analytical methods for the assessment of caffeine and its metabolites in a variety of matrices. We here report a sensitive and specific gas-liquid chromatographic method for caffeine analysis in human plasma and saliva and in xanthine beverages including coffee, tea, cola-flavored softdrinks and cocoa. This GLC method has the shortest runtime but one ever published for an analytical caffeine method and hence is very convenient for the routine analysis of large amounts of samples. The described method was put to the test in a pharmacokinetic pilot experiment.
Standards and reagents Caffeine (CAF) and theophylline (TP), both as the monohydrate, and tetrabutylammonium hydrogen sulphate (TBA\(\cdot\)HSO\(_4\)) were purchased from Janssen Chimica, Beerse, Belgium. Pentane, ethanol, dichloromethane, and sodium hydroxide (Merck, Darmstadt, F R G ) were of analytical grade. 1-Jodopentane (Merck) was of synthetical grade.

A CAF stock solution in ethanol (1 mg/ml) was diluted to prepare reference solutions of 100, 25, 5, and 1 μg/ml. The solutions were stable for at least 1 month when stored at 4°.

Synthesis of the internal standard 7-pentyl TP (7p-TP) was used as an internal standard (ISTD) and was synthesized by extractive alkylation of TP according to the directives given by Bonati et al [3]. To each of twenty 10 ml tubes 100 μl of a TP solution in ethanol (1 g/l) was added. After evaporation of ethanol (50°, dry nitrogen), 22 55 mg TBA\(\cdot\)HSO\(_4\) dissolved in 2 5 ml 0 1 N NaOH, 2 5 ml CH\(_2\)Cl\(_2\), and 0 5 ml 1-jodopentane were subsequently added. The tubes were tightly closed with a PTFE screw-cap and shaken for 1 h at 50° in a horizontal position. After centrifugation (5 min, 1000 g) the (lower) organic phases were transferred into fresh tubes and evaporated under dry nitrogen (40°). The dry residues were dissolved in 2 ml of pentane and left in an ultrasonic bath for 10 min. After centrifugation the clear pentane solutions were transferred to 20 new tubes and the pentane was evaporated. The residues were each taken up in 5 ml of ethanol to reach a concentration of approximately 26 μg 7-pentyl TP per ml (assuming the reaction yield is 100%). The ethanolic solutions were pooled, the 100 ml pool sufficed for a thousand assays and was stored at 4°.

Gas chromatograph and nitrogen-phosphorus selective detection A Hewlett Packard model 5710 gas chromatograph equipped with a nitrogen-phosphorus selective detector was used. The glass column (75 cm x 3 mm ID) was degreased by slowly leading a 80 ml boiling water-toluene (v/v = 50/50) mixture through it. Subsequently it was dried thoroughly, sylilated, and packed with 5% OV-17 on Gas-Chrom Q, 80-100 mesh. The packing was prepared by mixing Gaschrom Q coated with 10% OV-17 and with 3% OV-17 (Applied Science Laboratories Inc, Pensylvania, USA) in the ratio 2:5. The packing was deactivated by 5 injections with 3 μl N,O bis trimethylsilyl trifluoroacetamide (BSTFA, Pierce, Rockford, Illinois, USA), each injection time-spaced by 15 min. During
deactivation, helium was led through the column at a flow of 5 ml/min at an oven temperature of 100°.

Operating conditions were as follows: injection port temp. 250°, oven temp. 220°, detector temp. 300°, detector voltage 16 V, helium (carrier) flow rate 30 ml/min, hydrogen flow rate 3.0 ml/min, air flow rate 50 ml/min.

The detector output signal was led to a flow chart recorder (Model BD 8, Kipp & Zonen, Wormer, The Netherlands) and via an analog/digital converter (Model 18652 A, Hewlett Packard) to an electronic peak integrator (Model Silent 700 ASR, Texas Instruments).

Procedure. An aliquot of 100 μl of the ISTD solution was evaporated to dryness at 50° in a 10 ml test tube. Subsequently the sample to be analyzed, being either a 1.0 ml plasma or saliva sample or a beverage sample diluted 50 - 200 times to 1.0 ml with aqua dest, was added, followed by 50 μl 2 N NaOH and 7 ml of dichloromethane. The tube was then closed with a screw-cap and shaken for 10 min. After centrifugation (10 min, 1000 g) the organic layer was transferred into a clean tube and evaporated to dryness under a gentle stream of nitrogen at 40°. The residue was reconstituted in 50 μl of pentane. 2 μl aliquots were injected into the gas chromatograph.

Calibration was accomplished by using the procedure on prepared water or blank plasma standards to which known amounts of CAF had been added. Water standards were prepared in case of saliva or beverage analysis. Blank human plasma was supplied by the volunteer subjects after an abstinence period preceding each experiment. Before analyzing beverage samples or subjects' plasma or saliva samples, the plasma or water standards were analyzed and the peak areas of CAF and the ISTD were measured. A calibration curve was prepared by plotting the peak area ratio of CAF to the ISTD against the CAF concentration. A handcalculator programme calculated the regression line of the calibration curve. The beverage samples or the subjects' plasmas or salivas were then analyzed and the CAF to ISTD peak area ratios were determined. The CAF concentration in each sample was calculated according to the regression line.

Reproducability and recovery. The reliability of the method was tested by applying the procedure to 8 prepared 1 ml plasma or water samples spiked with 0.10 - 0.50 - 2.0 - 7.5 - 15.0 μg CAF. Mean values, standard deviations and ranges of the measured concentrations were determined. Coefficients of variation (CV) were obtained by dividing the standard deviation by the corresponding mean value for each concentration. Recoveries of CAF and its ISTD
were determined by injecting known aliquots of standard solutions of both compounds directly into the GC and comparing the resulting peak areas of each compound to those obtained after injection of worked-up samples.

Pharmacokinetic pilot experiment A healthy male non-smoking volunteer (23 years, 68 kg) who had abstained from coffee, thea, cola beverages, cocoa and chocolat products for 60 hours preceding the experiment was given 200 ml of freshly brewed coffee ('Zilvermerk', Douwe Egberts, Joure, The Netherlands) in a cup. The volunteer emptied the cup in 8 minutes, taking a draught every minute (time of the first draught was taken t = 0). The first blood sample was taken at t = 10 min. Then the subject drank another 200 ml coffee in 8 draughts, starting at t = 11 min. Further blood samples were taken at t = 21 - 29 - 37 - 46 - 55 min and at t = 1 1 - 1 3 - 1 8 - 2 8 - 4 3 - 6 8 - 12 4 - 23 4 hours. After the second cup of coffee, the subject was asked to brush his teeth thoroughly. 1.5 ml saliva samples were produced at t = 1 0 - 1 5 - 3 2 - 4 0 - 5 6 - 8 3 - 14 9 - 25 7 hours. Saliva production was not stimulated. Blood and saliva samples were centrifuged for 10 min at 1000 g. The plasma samples and the clear salivary supernatant were stored at -20°. Five coffee samples (5 μl), taken from each of the volunteer's two cups of coffee, were diluted to 1 ml with distilled water and frozen at -20° pending analysis. Analysis of all samples was performed two days after termination of the experiment.

5.4 RESULTS

Chromatography Figure 1 shows typical chromatograms of plasma, saliva and (diluted) coffee extracts. CAF (peak 2) is well separated from the ISTD (peak 3) and from a contaminant present in the ISTD solution (peak 1). The retention times are 0.9 min for this contaminant, 1.4 min for CAF and 2.7 min for ISTD. No other peaks of significant size are present in the chromatograms of either plasma, saliva, or beverage extracts. The runtime is as short as 3.3 min. The limit of analysis, defined as the lower concentration-limit that can be analyzed with a CV ≤ 10%, is 50 ng/ml.

Accuracy and recovery Table I lists spiked concentrations and measured concentrations together with their range and CV for a variety of CAF-
FIGURE 1 Chromatograms of (a) a plasma extract from our pharmacokinetic pilot subject after a 60 h abstinence period from all CAF-containing beverages, food and medications, (b) a plasma extract from the same subject at $t = 6.8$ h in the pilot experiment, the CAF concentration being 1.6 µg/ml, (c) a saliva extract from the subject after the 60 h abstinence period, and (d) an extract from the coffee after 200 times dilution, the resulting CAF concentration being 1.9 µg/ml. Peaks 1 = unidentified contaminant present in the ISTD standard solution, 2 = CAF, 3 = ISTD (7-pentyl theophylline)

concentrations in plasma and water. Measured values correlate within at most 6% with the spiked values. The CV is as low as 1.9% at a plasma CAF concentration of 15.0 µg/ml. The detection limit, defined as the injected amount of CAF yielding a peak signal to noise ratio of 2, is about 40 pg CAF, corresponding to a plasma concentration of 1 ng/ml.

The correlation coefficient $r$ averaged 0.9990 for an 8-point plasma regression line, 1 mL blank plasma or water samples being spiked with 0 - 0.5 - 1.0 - 2.0 - 5.0 - 8.0 - 10.0 - 15.0 µg CAF. The lowest $r$ value obtained in one month of almost daily analysis was 0.9971. We are unable to give a general equation for the regression line, because the slope of this line depends on
TABLE I REPRODUCIBILITY AND ACCURACY OF THE PROPOSED ANALYTICAL PROCEDURE AT VARIOUS CONCENTRATIONS OF CAFFEINE IN PLASMA AND WATER

<table>
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<tr>
<th>Spiked conc mg/l</th>
<th>Measured conc mg/l</th>
<th>Range of conc mg/l</th>
<th>CV %</th>
<th>R %</th>
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<td>1 9 91 9</td>
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</table>

the concentration of the ISTD solution and hence on the efficiency of the synthesis reaction, which varies somewhat between batches. Additionally, the slope of successively obtained regression lines using one particular batch gradually increases some 20% in the course of 4 - 6 weeks, the increase must be due to slow decomposition of the internal standard solution. It was ascertained that no decomposition products interfered with the CAF-peak. In fact, no decomposition peaks at all were observed after injection of an aliquot of the ISTD solution, no matter the age of the latter. It is unlikely that peak 1 in the chromatograms of fig 1 originates from an ISTD decomposition product. Peak 1 is already present in chromatograms of the ISTD solution immediately after synthesis of the ISTD, and its relative height to the ISTD peak doesn't increase or decrease significantly with aging of the solution. It was ascertained as well that the three dimethylxanthine metabolites of CAF did not co-elute with their parent compound, which would give rise to falsely elevated CAF concentrations. Paraxanthine, theobromine and TP were retained on the GC column after injection under operating conditions.
FIGURE 2 Plasma (*) and saliva (o) caffeine concentration versus time curves in the pharmacokinetic pilot subject, after swallowing a 400 ml-volume of freshly brewed coffee containing 153 mg of CAF.

The CAF recovery averaged 87.9 - 103.0 % depending on its concentration and the matrix, the recovery of the internal standard 91.3 ± 6.8 % in plasma and 93.1 ± 4.1 % in water (Table I). The tendency of the CAF recovery to diminish with increasing CAF concentration could reflect a limited solubility of CAF in the extraction fluid dichloromethane. However, this tendency was too weak to produce marked non-linearities in calibration curves in the CAF concentration range from zero to 7.5 μg/ml. The latter range normally covers the concentrations occurring after intake of up to 300 mg of CAF.

Pharmacokinetic pilot experiment The mean CAF concentration in the coffee drank by the volunteer was found to equal 384 ± 23 mg/l (mean ± SD, n=10). There was no significant concentration difference between the coffee in first and in the second cup, indicating that the rate of a possible thermal decomposition reaction of caffeine in freshly brewed coffee is too slow to be of importance in the 11 minutes time interval that separated the consumption of the two cups.
The total CAF dose in the 400 ml volume of coffee ingested by our volunteer thus amounted to $153 \pm 9$ mg. The CAF concentration vs time curves in the volunteer's plasma and saliva are given in figure 2. Absorption is seen to be rapid ($k_{abs} = 3.54$ h$^{-1}$), the plasma CAF concentration reaching its top 1 h after the first draught. The plasma elimination half-life $t_{1/2, p}$ is 3.8 h and the area-under-the-curve is 28.7 mg h/l. The mean body residence time of caffeine molecules equals 5.75 h. Assuming complete absorption of CAF from the gastro-intestinal tract [4,5], total clearance is 5.34 l/h and the distribution volume of CAF is 30.7 l in this subject. These values are consistent with the findings for healthy men in earlier investigations [4,5,6], in which analyses were performed with HPLC methods.

The saliva curve almost parallels the curve, its angle of decline is a little greater, reflected in a somewhat shorter saliva half-life compared to that in plasma $t_{1/2, s} = 3.6$ h. The mean saliva/total plasma caffeine concentration ratio between $t = 1$ h and $t = 25$ h was 0.79, which is in good accordance with the value of $0.79 \pm 0.02$ found by Zylber-Katz and coworkers in 12 healthy subjects [7] and with that of $0.73 \pm 0.08$ found by Hildebrandt cum suis in 6 healthy nursing women [8].

5.5 DISCUSSION

In caffeine analysis, as in the analysis of so many other drugs, the older non-chromatographical techniques, like the insensitive and insensitive UV spectrofotometrical measurements and the laborious radio-immuno-assays, have been replaced by rapid, specific and sensitive chromatographical assays. Many analytical procedures have been reported for the analysis of caffeine and related methylated xanthines and uric acids in biological fluids using straight-phase [9-13] or reversed-phase [3, 14-22] high-performance liquid chromatography (HPLC) or cation-exchange HPLC [23]. In all these methods, detection invariably is accomplished by monitoring the UV-absorbance of the eluent at either 250-260 nm or at 270-280 nm. Two until up to sixteen xanthines and/or uric acids are separately eluted in one run [21], runtimes varying from 2.5 min [3] until more than 45 min [20].

Published GLC assays for caffeine and related compounds in biological fluids typically cover the analysis of one or two di- or trimethylated xanthines in plasma/serum or saliva [3, 24-33]. Detection is accomplished by a
flame-ionization detector (FID) [24-29], a nitrogen-phosphorus detector (NPD) [30, 31] or by mass spectrometry (MS) [32, 33] GLC and HPLC xanthine assays rival in accuracy, in the higher concentration range (≥ 0.5 mg/l) the liquid chromatographic methods tend to be slightly more reproducible, which maybe is due to the more wide-spread use of automated injection facilities in LC, whereas in the lower concentration range the GLC methods yield superior results because of the higher sensitivity of especially FID and NPD compared to UV-absorbance detection. The GLC runtimes rarely exceed 10 minutes.

Unfortunately, the GLC caffeine assay having the shortest runtime (2 min) is afflicted with a non-linear shape of the regression line and with accordingly adverse reproducability characteristics [3]. Another, early published method employs an external rather than an internal standard, hampering reproducability as well [24].

Mass spectrometry as a detection technique for caffeine is less sensitive than either FID or NPD. This drawback can be partially compensated for by using a capillary GLC column in combination with splitless injection [32]. The GLC-MS method reported by Merriman et al. [33] however does not employ a capillary column and indeed is less sensitive than the former mentioned method [32] In addition, its specificity is insufficient, it probably co-determines caffeine and an endogenous compound, resulting in perceptible overestimations of caffeine amounts at concentrations lower than 1 μM (= 0.2 mg/l) [33].

The GLC-FID method for caffeine analysis developed by Gurtoo and Phillips [25] is characterized by broad peak shapes and at least one unidentified interference. The peaks of caffeine and its ISTD in the method of Perrier and Lear [26] elute on the tailing slope of a giant solvent/endogenous peak, a condition that doesn’t foster the accuracy and reproducability of the analytical results. The method published by Cohen et al. [27] suffers from an endogenous bump present in the chromatograms, whose late elution forces the runtime to be as along as 12 min. Gurtoo and Phillips [25] and Brazier and co-workers [30] do not specify analytical reliability data of their assays.

The GLC method reported here and the assays of Johnson et al. [28], Bradbrook et al. [29], Least et al. [31] and Floberg et al. [32] have in common well-resolved chromatograms with peaks ascending from baseline and descending back to it, no interferences from endogenous or exogenous compounds, a linear regression line over a wide concentration range, and excellent specificity, sensitivity and reliability characteristics. In addition, the reported method has an extremely short runtime (3.3 min), only the Bonati GLC method [3] is faster (20 min), but, as stated before, in other respects has adverse characteristics.
Because of the sensitivity of the assay, the starting volume of plasma or saliva, defined as 1.0 ml under 'Procedure', may easily be reduced to 100 μl or even less if the samples originate from neonates or small laboratory animals. Except to plasma, saliva or coffee the method may be applied to tea, cola-flavored soft-drinks and cocoa, but not to human urine.

Like in the assays developed by Johnson [28], Least [31] and Floberg [33] and their respective co-workers, in the method presented here the structural analogy between the ISTD and the xanthine compound to be analyzed is very close and certainly contributing to the beneficial characteristics of these assays.

In our laboratory the present assay has been used to determine the CAF concentrations in coffee and tea drank by six healthy volunteers and subsequently to elucidate the absorption and disposition kinetics of CAF in these subjects. Detailed data are reported elsewhere in this dissertation.

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6.1 INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) are closely related alkaloids (see figure 1) that occur in plants with wide geographical distribution and are part of man's dietary habit from time immemorial.

Coffee, the most important source of caffeine in the American and European diet is extracted from the fruit of Coffea arabica and related species. At least half the world consumes tea, prepared from the leaves of Thea sinensis and containing caffeine, small amounts of theobromine and traces of theophylline. Cocoa and chocolat, from the seeds of Theobroma cacao, contain theobromine and some caffeine. Cola-flavored drinks contain 100 - 150 mg/l caffeine, partly so because the nuts of Cola acuminata are processed in these drinks, and partly because of the addition of caffeine as such in their production.
Caffeine, theophylline and theobromine share in common a variety of pharmacological actions [1]. They stimulate cardiac muscle, relax smooth muscle (notably bronchial muscle), act on the kidney to produce diuresis, and they stimulate the central nervous system (CNS). The latter action is the basis for the popularity and the mass-consumption of the xanthine-containing beverages.

In adults, the methylxanthines are mainly metabolized in the liver, undergoing successive N-demethylation and oxidation steps, giving rise to xanthines and uric acids containing less than 3 methyl groups [2]. Also the 5-membered xanthine ring may be metabolically cleaved to yield substituted uracils [3]. A detailed scheme of caffeine metabolism is included in this dissertation (Chapter 13, figure 1).

After an oral or intravenous dose of caffeine, the dimethylxanthines are detectable in human plasma. However, the concentrations of paraxanthine (1,7-dimethylxanthine, see also figure 1) outweigh those of theobromine and theophylline, indicating the 3-demethylation pathway to be the most important initial step in caffeine metabolism [4,5].

FIGURE 1. Structural formulas of caffeine and the three dimethylxanthines.
In the course of a study of the pharmacokinetics of caffeine and the rate and extent of its biotransformation into paraxanthine, we have developed a reversed-phase HPLC method suited to monitor caffeine and paraxanthine in the range of concentrations that occur in human biological fluids after administration of a caffeine dose in the order of magnitude of 100 mg. It probably is also applicable to the analysis of theobromine and theophylline in biological fluids, however, the accuracy of the presently proposed method was not investigated for theobromine and theophylline analysis. The method may also be applied to xanthine beverages.

To demonstrate its appropriateness, it was applied to determine caffeine levels in tea drank by a volunteer subject, and to those of caffeine and paraxanthine in samples of body fluids produced by this volunteer.

**TABLE I. COMPOUND ABBREVIATIONS EMPLOYED**

CAF = caffeine (1,3,7-trimethylxanthine)
PX = paraxanthine (1,7-dimethylxanthine)
TP = theophylline (1,3-dimethylxanthine)
TB = theobromine (3,7-dimethylxanthine)
ISTD = internal standard (antipyrine)

6.2 MATERIALS AND METHODS

Chromatography A Hewlett Packard high performance liquid chromatograph (model 1081B) equipped with a variable-volume injector was used. The stainless steel column (15 cm x 4.6 mm ID) was packed in our laboratory with LiChrosorb® RP-8, particle size 5 μm (Merck, Darmstadt, F.R. G.). The oven temperature was chosen 40°C and the injection volume 15 μl. The mobile phase, consisting of 5 mM acetic acid (pH = 3.5) / methanol / acetonitrile / tetrahydrofuran (90 4 4 2, v/v) was delivered at a flow of 1.0 ml/min, the resulting pressure drop being 150 Bars. The eluate was monitored at 254 nm UV. The detector signal was displayed on a flow chart recorder (Kipp & Zonen model BD-8, Wormer, The Netherlands) and was as well transferred to a Hewlett Packard 3353 Lab Auto System for the purpose of electronic peak integration.
Reagents CAF and TP were purchased both as the monohydrate from 'De Onderlinge Pharmaceutische Groothandel', Utrecht, The Netherlands. TB was obtained from Jansen Chimica, Beerse, Belgium, and PX from Fluka, Buchs, Switzerland. The internal standard (ISTD) antipyrine, and trivial solvents and chemicals, all of analytical grade, were obtained from Merck, Darmstadt, West-Germany. Doubly distilled water was used for the preparation of the HPLC mobile phase and a phosphate buffer solution.

Standards and solutions Stock solutions of CAF and PX in ethanol (1 00 mg/ml) were diluted to produce working solutions of 100, 10 and 1 0 μg/ml. The solutions do not show any signs of decomposition up to at least one month after preparation if stored at 4°. An ISTD stock solution in ethanol (1 mg/ml) was diluted to produce a working solution of 50 μg/ml. The ISTD solutions were stable for at least 6 months after preparation when stored at 4°.

A 0 5 M phosphate buffer solution was made up by dissolving the appropriate amount of disodium hydrogen phosphate in aqua bidest followed by adjustment of the pH to 7.9 by addition of a few drops of concentrated hydrochloric acid.

Procedure Xanthine beverages were diluted a factor 100 (coffee) or 40 (tea) in case of CAF analysis, but were not diluted in case of dimethylxanthine analysis. A 100 μl aliquot of the ISTD working solution was pipetted into a 10 ml extraction tube and evaporated to dryness at 45° under a gentle stream of dry nitrogen. A 1 ml plasma, saliva or (whether or not diluted) beverage sample, 1 ml of phosphate buffer and 6 ml of dichloromethane was successively added to the tube which was then closed with a poly-tetrafluoroethylene (PTFE) screw-cap and shaken mechanically in a horizontal position for 10 minutes, followed by centrifugation (1000 g, 10 min). The aqueous and protein layers were removed by suction, the organic layer was transferred to a clean tube and evaporated to dryness at 40° under nitrogen. The residue was taken up in 100 μl of the HPLC mobile phase. An aliquot of 15 μl was injected onto the column and run under the conditions described under 'Apparatus'.

Calibration was accomplished by using the procedure on a series of 11 prepared water or blank plasma standards which were spiked with mounting, known amounts of CAF and PX (0 - 10 μg/ml for both compounds). Water standards were prepared in case of saliva and beverage analysis.

Calibration curves for CAF and PX were prepared by plotting the peak area ratio of CAF to ISTD and of PX to ISTD against the spiked concentration.
of CAF and PX, respectively. The least-squares regression line of these 11 calibration points was calculated by a handcalculator program. The concentrations of CAF and PX in unknown samples were calculated according to this regression line.

Pharmacokinetic pilot study. A healthy female non-smoking volunteer (age 24 years, weight 68 kg), free of medication at the time of the study participated in the following experiment. After a 60 hours abstinence period from coffee, tea, cola, chocolate and caffeine-containing medications and after an overnight fast she produced a blank sample of blood and saliva. Then she drank 400 ml of freshly prepared tea. The mid-point of ingestion of the 400 ml aliquot was taken as zero-time. Blood-samples (8 ml) were taken at t = 9 - 18 - 29 - 37 - 47 and 56 min and at t = 11 - 14 - 16 - 19 - 26 - 36 - 49 - 78 and 156 h. At t = 10 h, the volunteer was requested to brush her teeth and rinse her mouth thoroughly. Samples of saliva (10 - 20 ml) were produced at t = 13 - 18 - 25 - 37 - 48 - 59 - 66 - 80 and 157 h. Ten aliquots of 25 µl each were taken from the tea just before ingestion and diluted to 1.0 ml with aqua dest for the purpose of CAF analysis. Ten other tea-samples (10 ml) were not diluted and analysed for their dimethylxanthine concentration. Blood and saliva samples were centrifuged for 10 min at 1000 g to produce blood plasma and clear salivary supernatant. Biological and beverage samples were stored at -20° pending analysis, which was performed within 7 days after termination of the experiment.

6.3 RESULTS

Chromatography. Figure 2 shows representative chromatograms of a variety of commonly processed kinds of samples. The peaks of the xanthines and the ISTD are well-resolved, except for the PX and TP peaks which overlap to some extent. This overlap may be eliminated by the use of a 25 cm-column or by an increase of the volume ratio of the aqueous phase and the organic modifier in the mobile HPLC-phase to 92½ / 7½ vol % instead of 90 / 10 vol %. We let the run time advantage associated with the latter mobile phase composition preponderate before complete peak separation offered by the former one, as the peak overlap did not interfere seriously with good analysis (see 'Discussion')
FIGURE 2 Chromatograms of (a) a column resolution test mixture, consisting of a 1 ml water sample spiked with roughly equal amounts (by weight) of CAF, each of the 3 dimethylxanthines and ISTD, (b) an undiluted tea sample, (c) a plasma sample and (d) a saliva sample taken from the pilot subject almost simultaneously at about $t = 1.35$ h after ingestion of 60.4 mg of CAF as tea, (c') a plasma and (d') a saliva sample both taken from the subject just before tea consumption. Peaks $1 = TB$, $2 = PX$, $3 = TP$, $4 = CAF$, $5 = ISTD$.

Chromatograms of plasma and saliva showed a similar appearance, as those of coffee and tea. Retention times are 2.7 min for TB, 3.4 min for PX, 3.8 min for TP, $5.1$ min for CAF and $9.3$ min for the ISTD. As there are no endogenous peaks eluting after the ISTD, the chromatographical runtime is 11 minutes. The 'blank' chromatograms (figure 1 c'/d') show the peak of the endogenous (desmethyl) uric acid at about 2 min, and a small unidentified lump at 5.75 min. The latter was invariably present in the body fluids of the pharmacokinetic subjects, although hardly detectable in the case of many of them. It interfered markedly with a precise CAF-peak area integration at CAF-concentrations $\leq 0.2$ $\mu g/ml$. At these low levels, the CAF to ISTD peak height ratio was used to determine the exact CAF concentration. The CAF peak height was not affected by the lump at any concentration above the detection limit.
### TABLE II. TOTAL RECOVERY AND ACCURACY OF THE ASSAY

CV = coefficient of variation, R = total recovery, n = number of samples

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<td>98.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>2.0</td>
<td>96.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>ISTD</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>101.0</td>
<td>60</td>
</tr>
</tbody>
</table>

\*At this concentration the peak height ratio of CAF to ISTD was used rather than the area ratio.

Total recoveries and accuracy. The total recoveries of CAF, PX and the ISTD are listed in the 'R'-column of Table II. The total recovery of these compounds has a value between 95 and 106% in the concentration range investigated.

Calibration curves are linear up to concentrations of at least 9 µg/ml of CAF and the three dimethylxanthines in plasma and saliva. Correlation coefficients mostly exceeded 0.999 and the y-intercepts were not significantly different from zero for any of the xanthines. The precision of the assay procedure is more than satisfactory, measured CAF and PX concentrations deviating no more than 4% from the spiked concentrations (see Table II). The CAF and PX concentrations which are determinable with a coefficient of variation (CV; Table II) less than 10% nicely covers the the range of CAF and PX plasma and saliva levels occurring in man after consumption of a dose of CAF as small as 60 mg (see figure 3).
FIGURE 3 Concentration vs time profiles of caffeine (*) in plasma and (o) in saliva, and of its main metabolite paraxanthine again (△) in plasma and (△) in saliva, obtained from a healthy female volunteer after drinking a 400 ml volume of freshly brewed tea containing 60.4 mg of caffeine.

Pharmacokinetic pilot study Analysis of the diluted and undiluted tea samples revealed the following amounts of xanthines to be present in the 400 ml-volume of tea drunk by our volunteer subject: 60.4 ± 1.1 mg of CAF, 2.05 ± 0.04 mg of TB, and 0.08 ± 0.01 mg of TP. So, contrary to popular belief this brew of tea, as well as brews of other brands of tea we analyzed, hardly contained any TP. No PX at all was found (see also figure 2b).

The CAF and PX concentrations in the plasma and saliva of the volunteer subject are depicted as a function of time in figure 3. The CAF peak plasma concentration is reached after approximately 45 min, indicating rapid CAF absorption in the GI tract. After the peak time the CAF plasma and saliva concentration profiles decrease in parallel with a biological half-life of 3.8 hours. The saliva to total plasma CAF concentration ratio averages 0.72 in this subject.
The CAF plasma concentration-time curve was fitted to a biexponential equation; from the fitted parameters the following pharmacokinetic quantities were calculated: $\text{MRT} = 5.14 \text{ h}$, and, assuming complete systemic availability [6,7], $\text{CL} = 6.83 \text{ l/h}$, $V_dss = 35.1 \text{ l}$.

These values agree with the values found for the other pharmacokinetic subject (GS, see previous chapter) and with earlier published kinetic data for CAF (see references in the previous chapter).

The PX plasma and saliva concentration profiles are highest at $t = 4 \text{ h}$. These curves have a typical convex 'gamma' shape, indicating that the dominant characteristic times 'τ' in the disposition (transport) functions of the parent compound (CAF) and the metabolic daughter (PX) are similar. The plasma curves of CAF and PX intersect at $t = 10\frac{1}{2} \text{ h}$. The PX concentration ratio in saliva to total plasma is 0.60. Hence it appears that PX cumulates in saliva to a lesser extent than CAF does, which indeed was to be anticipated on the basis of its stronger plasma protein binding compared to CAF [9].

6.4 DISCUSSION AND CONCLUSIONS

The reported assay procedures for the analysis of CAF and PX in biological fluids and xanthine beverages is rapid, sensitive and selective. It is composed of a single dichloromethane extraction of the sample followed by evaporation of the extraction solvent and reversed-phase high performance liquid chromatography of the concentrated residue. Its limit of analysis, defined as the threshold concentration value at which CV = 10% is 0.1 μg/ml for both CAF and PX departing from a 1 ml sample volume.

The assay procedure may very well be valid for analysis of TB and TP in biological fluids and beverages. The recovery and accuracy data for the latter compounds were not established, but are likely comparable to those of CAF and PX (Table I). If analysis of both PX and TP is desired, measures should be taken to eliminate the overlap existing between the PX and TP peaks (fig. 1). Suggestions for such measures have been proposed in the section 'Results-chromatography'. If either PX or TP is to be analyzed, and if this compound is present in excess of the other (in our case concentrations of PX outweigh those of TP), then such measures are superfluous since in these circumstances the minor peak does not interfere with the major one (e.g., see figure 1c/d).
Though the chemical structure of the internal standard antipyrine hardly bears any resemblance to that of the xanthines, the reproducability characteristics of the described method are evidently good. A synthetical xanthine widely employed as an ISTD in literature on caffeine assay: β-hydroxyethyl theophylline, was abandoned as its peak and the PX-peak appeared to overlap in the chromatograms of the present method.

Taking the rapidness, sensitivity, accuracy and applicability features together, the present method compares with the assays published by Wahllander et al. [10], Schulz et al. [11], Tse and Szeto [12] and Muir et al. [13]. Several assay methods for the analysis of CAF and the dimethylxanthines have been published in which the peaks of PX and TP overlap heavily (e.g. [14]), are stated to coincide, or in which there is made no mention of PX [15-18] in spite of the presence of a CAF-peak. In the latter case the apparently missing PX-peak again must be suspected to hide under the peak designed 'TP', so there is every reason to be sceptical of the 'theophylline'-levels reported!

A final remark concerns the relative abundances of CAF and the dimethylxanthines in the coffee and the tea ingested by the volunteers partaking in the pharmacokinetic experiments described in the section of this dissertation dedicated to the clinical pharmacokinetics of caffeine.

The coffee we brewed in the course of the above experiments (brand: Douwe Egberts Zilvermerk, Douwe Egberts, Joure, The Netherlands) as analyzed by our method was found to contain CAF and TB in a ratio of approximately 1000 to 1. No TP was found.

The tea we served to our pilot subject and to the other subjects (brand: Pickwick Chinese melange, Douwe Egberts) contained CAF, TB and TP in a ratio of roughly 1000 to 20 to 1. No PX was found.

In theory, dimethylxanthine competition with CAF for the hepatic microsomal enzyme systems could become manifest at total methylxanthine concentrations high enough to render methylxanthine metabolism capacity-limited. As the total abundance of the dimethylxanthines in coffee and tea constituted no more than 0.1 and 2 % of the CAF abundance, respectively, the presence of this small amount of dimethylxanthines is unlikely to have contributed significantly to any occurrence of capacity-limited CAF disposition kinetics in our experimental volunteers.
6.5 REFERENCES

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Chapter 7

SELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC
METHOD FOR ESTIMATING QUININE LEVELS IN BIOLOGICAL
FLUIDS

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7.1 SUMMARY

A new reversed-phase high-performance liquid chromatographic method
employing fluorescence detection for the rapid quantification of plasma and
urine levels of quinine is described The method involves internal standardi­
ation, an enzymatic hydrolysis of quinine glucuronide in urine samples and
extraction of alkalized serum or urine The organic extract is evaporated
and the residue reconstituted in a small volume of the mobile phase 10 μl
aliquots are injected onto the column The effluent is monitored using fluo­
rescence detection The minimum determinable concentration with a coefficient
of variation of 10 % is 0.05 μg/ml for plasma samples and 0.1 μg/ml for urin­
ary samples

Advantages of the present method are selective and reliable detection
without interferences from endogenous plasma or urine compounds or detergents
derived from the tube-cleaning machine, a very short chromatogram runtime (5
minutes), the employment of relatively nontoxic extraction- and mobile phase
solvents and isocratic delivery of the mobile phase, suiting the method to be
run on any HPLC equipment The method is convenient for the routine analysis
of large quantities of samples and is facile adaptable to the monitoring of a
number of other compounds, e.g. quinidine
Quinine (Q), (figure 1-I), a member of the cinchona alkaloid family, has been widely used for the treatment of falciparum malaria. Though largely replaced by synthetic antimalarial drugs, including mecapnne and choroquine, Q is still the only consistently effective drug for severe chloroquine-resistant falciparum malaria [1]. Besides, Q is used as a bitter principal in the flavoring of carbonated soft drinks.

Q is metabolized in man by oxidation of the quinoline and quinuclidine moieties, resulting in metabolites with a phenolic and nonphenolic character, respectively [2]. Q metabolism parallels the metabolism of its isomer quinidine, but the relative quantities in which the various metabolites are formed in man differ significantly for the antimalarial and the antiarrhythmic agent, as well as the amount of drug excreted unchanged in the urine [2, 3].

Few definite knowledge is available of the relative amounts in which the various Q metabolites are formed in man. However, some important Q metabolites, whose structural formulas are shown in Figure 1, appear to be Q-N-oxide (II), 3-OH-Q (III) [2, 4], 6'-OH-cinchonine (O-desmethyl Q) (IV) [4], and the keto-enol tautomer pair 2'-quininone (V) - 2'-OH-quinine [2, 4].

A limited number of Q assay procedures has been reported in modern literature, e.g. [4-9]. In contrast, quinidine, which is still in widespread therapeutic use, has been the object of numerous assay procedures, e.g. [6-21], many of which can be applied unmodified, or are easily adapted, to Q assay. The Q and quinidine assay procedures include plasma protein precipitation combined with fluorescence [11], where the background fluorescence may be determined by selectively quenching cinchona alkaloid fluorescence with chloride ions [12], single extraction-fluorescence [5, 13] and double extraction-fluorescence [14]. These fluorescence methods all suffer from fluorescent contributions from either metabolites or unknown constituents of the plasma, often leading to spurious estimates of Q concentrations. In clinical analysis, co-determination of the metabolites can be essential because the metabolites may have pharmacological activity too. In determining pharmacokinetics however, it is a prerequisite to measure merely Q.

Thin-layer chromatographic (TLC) methods [10, 15, 16] can be made very specific but are tedious and time-consuming and hence inconvenient for the routine processing of large numbers of samples.

The most recently published methods utilize high-performance liquid chromatography (HPLC), offering rapidity, specificity, and sensitivity. These
FIGURE 1 Structural formulas of quinine (I), its metabolites quinine N-oxide (II), 3-hydroxy-quinine (III), 6'-hydroxy-quinine (O-desmethyI-quinine) (IV), 2'-quininone (V), and the internal standard methaqualone (VI)

methods indicate the use of a normal-phase column with alkaline extraction and either UV detection [9] or post-column acidification and fluorescence detection [5]. Another possibility is the use of a reversed-phase column with direct injection of plasma [17] or urine [18], injection of a supernatant obtained after plasma protein precipitation and centrifugation [19], or injection of an aliquot of residue reconstituted in the mobile phase after alkaline extraction and evaporation to dryness [8, 20, 21]. Both UV and fluorescence detection may be employed.

The vast majority of the HPLC methods referred to are applicable to either only urine, only plasma/serum or only suspensions of pharmaceutical preparations (capsules or tablets). Some of them utilize a multiple solvent delivery system to achieve gradient elution or a post-column mixing chamber to acidify the eluent. The selective and straightforward HPLC procedure described here is suitable to all kinds of biological samples and is characterized by isocratic solvent delivery. The method is aimed for rapid routine analysis of large amounts of samples.
Apparatus A Hewlett Packard HP 1081B high-performance liquid chromatograph equipped with a variable-volume injector was used. The stainless-steel column had a length of 15 cm and an internal diameter of 4.6 mm, and was packed with LiChrosorb RP-8, particle size 5 μm (Merck, Darmstadt, FRG), in our laboratory. The oven temperature was 40°C and the injection volume was 10 μl. Q and the internal standard were measured with a fluorescence spectrometer (Perkin-Elmer Model 3000), the excitation wavelength was 320 nm (slit 15 nm) and the emission wavelength was 355 nm (slit 20 nm). The mobile phase, consisting of 85 mM acetic acid buffer pH = 4.1 and tetrahydrofuran (THF) (60:40) was delivered isocratically at a rate of 1.0 mL/min, the resulting column head pressure being 220 bars. The mobile phase constituents were separately filtered, the acetic acid buffer through a 0.45 μm filter and THF through a 1.0 μm filter (Millipore), then mixed and degassed thoroughly in a Model 220 sonifier (Bransonic) at room temperature. Peak areas were determined with the aid of a Hewlett-Packard 3353 Lab Auto System.

Reagents Q was used as the diquinine sulfate dihydrate (Q₂·H₂SO₄·2H₂O). The internal standard (ISTD) metaqualone was supplied by 'De Onderlinge Pharmaceutische Groothandel', Utrecht, The Netherlands. β-Glucuronidase was obtained from Sigma Chemicals, St Louis, MO, USA. Diethyl ether, acetic acid, sodium acetate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, ethanol and tetrahydrofuran were all of analytical grade (Merck, Darmstadt, FRG), diethyl ether was distilled shortly before use. Buffers were made up in distilled water.

Standard solutions A stock solution of Q in ethanol at a concentration of 1g/l (as free base stable for at least two months at 4°C) was diluted to prepare standard solutions of Q at 100, 10 and 1 mg/l in ethanol. Internal standard solutions were prepared in ethanol at a concentration of 1 g/l for the internal standardization of plasma samples and at a concentration of 10 g/l for the internal standardization of urine samples. A 5 M sodium hydroxide standard solution was prepared in distilled water.

Procedure Until analysis, plasma and urine were stored at -20°C in the dark. Analysis was carried out avoiding direct irradiation by sunlight (see 'Photo-desintegration of Q'). In an extraction tube with PTFE screw-cap, 100
μl of ISTD standard solution (1 g/l for plasma samples, 10 g/l for urine samples) were pipetted and evaporated to dryness under a gentle stream of dry nitrogen at 50°. Aliquots of 1.0 ml of plasma or urine, 100 μl of sodium hydroxide standard solution and 7 ml of ether-dichloromethane (2:1) were successively pipetted into the extraction tube. The tube was closed with a screw-cap and shaken mechanically for 10 minutes, followed by centrifugation at 800 g for 12 minutes. The organic layer was transferred to another tube and evaporated to dryness under a gentle stream of air at 40°. The residue was reconstituted in 0.1 ml of the mobile phase (1.0 ml for urine). An aliquot of 10 μl of this solution was injected into the high-performance liquid chromatograph.

Calibration was accomplished by using the procedure on ten prepared 1 ml plasma or urine standards to which known amounts of Q (0 - 10 μg for plasma, 0 - 100 μg for urine standards) had been added. Before analyzing subjects' samples, the standard samples were analyzed and the peak areas of the Q and the ISTD peaks were measured. For each standard sample the Q peak area was divided by the ISTD peak area to obtain the ratio. A calibration curve was prepared by plotting the peak area ratio against the Q concentration. A basic programme calculated the regression line of the calibration curve. The subjects' samples were analyzed and the peak area ratios were determined. The Q concentration in each sample was calculated according to the regression line.

Analysis of Q glucuronide in urine Every aliquot of urine from a volunteer having taken Q orally (see 'Pharmacokinetic pilot study') was divided into two portions. In one portion free Q was determined as described under 'Procedure'. In the other portion the total amount of free Q and Q glucuronide was determined in the following way.

To an extraction tube containing an amount of internal standard (250 μl of a solution of 1 g/l, evaporated to dryness) were added 250 μl of urine and 250 μl of 0.2 M phosphate buffer (pH = 6.8) containing 500 units of β-glucuronidase. The extraction tube was closed and the contents were incubated for 4 hours at 37° in a heating block. After chilling to room temperature, the sample was processed further as described under 'Procedure' and measured as described under 'Apparatus'. A calibration curve was prepared by spiking ten 250 μl standards of blank urine with known amounts of free Q since Q glucuronide was not available to us.

Photo-desintegration of Quinine A solution of 20 mg/l Q in mobile phase solvent was spread over four Erlenmeyer flasks. Three of these flasks were exposed to light. The first flask was exposed to standard laboratory lighting.
(fluorescent lights Philips TL 40W/34 'de Luxe' at a distance of 0.40 m) The second flask was exposed to daylight without direct irradiation by the sun and the third flask was exposed to direct sunlight. The fourth flask served as a reference and was stored in the dark. All flasks were closed tightly to prevent evaporation of the solvent.

At various times a 1 mL aliquot of each flask was transferred to a vial and 10 μL of this was injected onto the column. Q peak areas of the three light-exposed solutions were divided by the corresponding peak area of the dark-stored solution. The peak area ratio was plotted against the time of exposure for each of the three light-exposed solutions. Photo-desintegration half-lifes \( t_{1/2}\),photo of Q and the ISTD in the three light-exposed solutions were determined.

Pharmacokinetic pilot study A healthy male volunteer (23 years old, weight 77 kg) refrained from drinking Q-containing beverages for one week. After production of a blood sample and a urine sample the volunteer ingested 107.7 mg Q in a gelatine capsule after a sober breakfast at 9:50 a.m. \((t = 0)\). 5 mL blood samples were taken at \( t = 0, 3, 6, 9, 12, 21, 30, 42, 51, 61, 71, 117, 247, 313\) hours. During this period ten urine samples were collected after spontaneous voiding.

7.4 RESULTS

Chromatography Figure 2 represents chromatograms of plasma and urine samples produced by the pilot volunteer before and after Q intake. The 'blank' chromatograms \((a_2\) and \(b_2)\) reveal that endogenous plasma and urine constituents do not interfere with the peaks of Q and the ISTD. Urine chromatogram \(b_1\) shows a peak of an unidentified Q metabolite (probably being 3-OH-Q, see 'Discussion') which in low concentrations also was observed in the volunteers' plasma, but not until several hours after dosage. Retention times are 1.9 min for the unidentified metabolite, 2.4 min for Q and 4.0 min for the ISTD.

Procedure For plasma Q concentrations from 0 to at least 10 μg/mL, there is a linear relationship between the peak area ratio of Q to internal standard (Y) and the plasma Q concentration (X) as given by the equation

\[
Y = 0.878 X - 0.0094 \quad (r = 0.999, \quad n = 10)
\]
FIGURE 2 Chromatograms of (a1) a plasma extract of a healthy male volunteer 1 20 h after intake per os of 107 7 mg quinine base, (a2) an extract of blank plasma, (b1) an extract of a urine aliquot (195 ml), voided 2 23 h after quinine administration, (b2) an extract of blank urine, (c) a 20 mg/l solution of quinine in HPLC mobile phase, exposed to direct sunlight during 6 h, (d) the latter solution, exposed to direct sunlight for 24 h Peaks: 1 = unidentified Q metabolite(s), 2 = quinine, 3 = internal standard (methaqualone), 4 = photo-degradation product of quinine.

For urine concentrations from 0.0 to at least 100 μg/ml, a linear relationship between the peak area ratio of Q to internal standard (Y) and the urinary Q concentration (X) is given by.

\[ Y = 0.0724 \times X - 0.0176 \quad (r = 0.999, n = 10) \]

Using blank human plasma and urine, the overall recoveries of Q and the ISTD always were in excess of 90%, the exact values are indicated in Table I. Within-day assay reproducibility and accuracy as a function of plasma and urine Q concentrations are given in Table II. The limit of analysis (LA), defined as the concentration giving rise to a coefficient of variation (CV) of ≤ 10 % (n = 10), is approximately 0.05 μg/ml for plasma and 0.1 μg/ml for urine, as Table II shows.
The LA values can be lowered further by increasing the injection volume or by decreasing the volume of the mobile phase in which the residue is reconstituted after evaporation to dryness. At concentration levels exceeding ten times the LA the CV is less than 3%. The limit of detection (LD), defined as the injected quantity giving rise to a signal that is twice the noise level (S/N = 2), is 50 pg Q. The range of determinable concentrations nicely covers the drug levels occurring after intake of a relatively small amount of Q (±100 mg) by a volunteer subject, as the pharmacokinetic pilot study revealed.

Photo-desintegration of Quinine. Figure 2c and d represent chromatograms of Q having been exposed to direct sunlight during 6 hours and 24 (4x6) hours, respectively. A degradation peak (peak 4) is observed having a retention time of 2.7 minutes. The same degradation peak was observed in case of exposure to shaded daylight and laboratory lighting. Desintegration of Q was found to obey first-order kinetics to a reasonable approximation whether exposed to sunlight, shaded daylight or laboratory lighting, and consequently in each of these cases a photo-desintegration half-life \( t_{\frac{1}{2},\text{photo}} \) could be calculated (Table III). The values of this half-life naturally depend on the quality of the particular lightsources to which the Q solutions are exposed, and probably on the nature of the solvent as well. The values shown in Table III therefore should be viewed merely as an indication of the order of magnitude of the rate of the degradation process.

Irradiation by direct sunlight should thus be strictly avoided while processing Q samples. Dark-brown glassware offers sufficient protection against daylight if direct solar irradiation is excluded, while no special precautions are necessary if samples are exposed only to internal laboratory light sources of the kind we investigated.

Pharmacokinetic pilot study. A computer fit to a sum of three exponentials of the volunteers plasma Q concentration versus time is plotted in figure 3 (top). Two peak plasma levels were found: 0.92 µg/ml at 2.1 h and 0.73 µg/ml at 6.1 h. The curve fitting program was provided with a second dose statement in order to obtain a satisfactory fit of the dual-peaked curve, the ratio of the two Q doses and time of administration of the 2nd dose were optimized iteratively. The area under the plasma curve (AUC) was calculated as 11.2 mg h/l. The absorption constant \( k_a \) was determined as 0.73 h⁻¹, the elimination half-life \( t_{\frac{1}{2}} \) as 8.73 h, and the mean residence time (MRT) as 11.6 h.
TABLE I  ANALYTICAL RECOVERY OF QUININE AND ITS INTERNAL STANDARD AT VARIOUS
CONCENTRATIONS IN PLASMA AND URINE

\[ R_{\text{plasma}} = \text{total recovery from blood plasma}, \quad R_{\text{urine}} = \text{total recovery from urine}, \quad n = \text{number of analyses} \]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>( R_{\text{plasma}} )</th>
<th>( R_{\text{urine}} )</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>0.5</td>
<td>94 ± 2</td>
<td>92 ± 3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>93 ± 1</td>
<td>93 ± 2</td>
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<tr>
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<td>100</td>
<td>99 ± 2</td>
<td>97 ± 2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
<td>10</td>
</tr>
</tbody>
</table>

TABLE II  WITHIN-DAY ACCURACY OF THE PROPOSED ASSAY AT VARIOUS CONCENTRATIONS
OF QUININE IN PLASMA AND URINE

\[ CV = \text{coefficient of variation}, \quad n = \text{number of analyses} \]

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Spike conc ( \mu g/ml )</th>
<th>Measured conc ( \mu g/ml )</th>
<th>Range ( \mu g/ml )</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.20</td>
<td>0.18 - 0.23</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.51</td>
<td>0.49 - 0.54</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2.05</td>
<td>1.98 - 2.15</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Urine</td>
<td>0.100</td>
<td>0.098</td>
<td>0.086 - 0.117</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.98</td>
<td>0.95 - 1.02</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>5.02</td>
<td>4.81 - 5.18</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>20.00</td>
<td>20.10</td>
<td>19.26 - 20.60</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

TABLE III  PHOTO-DESIINTEGRATION HALF-LIFES OF DISSOLVED QUININE EXPOSED TO
VARIOUS SOURCES OF IRRADIATION

<table>
<thead>
<tr>
<th>Source of irradiation</th>
<th>Daylight (bright sun)</th>
<th>Daylight (shaded)</th>
<th>TL tube (at 40 cm distance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/2,\text{photo}} )</td>
<td>8 ± 2 hours</td>
<td>30 ± 0.5 days</td>
<td>60 ± 9 days</td>
</tr>
</tbody>
</table>
FIGURE 3 (Top) Quinine plasma concentration versus time profile in a healthy male volunteer after ingestion of a gelatine capsule containing 107.7 mg of quinine base. (Bottom) Cumulative renal excretion of (a) unchanged quinine, and (b) the sum of unchanged and conjugated quinine (the latter expressed as mg of free quinine base) as a function of time in the volunteer subject.
Q was excreted as the glucuronide conjugate to a minor but significant extent in this subject. The cumulative renal excretion of Q in 31 hours amounted to 18.42 mg (figure 3, bottom, curve a), whereas after incubation of the urine with β-glucuronidase 23.80 mg of free Q base was recovered (figure 3, bottom, curve b). The 5.38 mg increment (5.0% of the ingested dose) represents the amount of Q excreted as the conjugate. The MRT of Q as calculated from the renal excretion data (figure 3, bottom, curve a) is 11.3 h, a value virtually identical to the one calculated from the plasma data.

As the second peak was reached about 2 hours after the subject's lunch, the intake of food may have stimulated renewed absorption of so far unabsorbed Q. Alternatively, because Q metabolism apparently includes conjugation, the dual plasma peak pattern could be the result of enterohepatic recirculation of Q. Again, the subject's lunch could have been the trigger of biliary secretion of Q-glucuronide, setting the recirculation going. It was not possible on the basis of the given observations to assign truth to one of both hypotheses.

7.5 DISCUSSION

The method presented here for the analysis of Q in biological fluids has been used to determine the pharmacokinetics of Q after oral and intravenous administration in 7 volunteers. Apart from its beneficial selectivity and sensitivity characteristics, the method is quite suitable in toxicological respect. It utilizes ether and dichloromethane as extraction solvents instead of the widely employed solvent toluene, which is far more toxic and should not be handled in a routine fashion. THF is used as a modifier of the mobile phase replacing the cheaper but highly toxic acetonitrile. By this choice of organic solvents time-consuming safety precautions or special expensive adaptations of the chromatographic equipment are rendered superfluous. In fact these toxicological considerations have led us to develop a new Q assay method, because the ventilation characteristics of the operating room of our HPLC equipment did not allow us to use toluene or acetonitrile.

An important contribution to the selectivity of the method, the separation of Q metabolites, deserves a brief word. The polarity of the N-oxide group probably results in a poor co-extraction of Q-N-oxide (figure 1-II) into the apolar extraction phase and, in so far as extracted, in an excellent separation from Q on the column. Phenolic metabolites, including 2'-OH-Q, 3'-OH-Q,
6'-OH-cinchonine (IV) and the vast majority of the minor polyhydroxylated metabolites bear a unity negative electric charge at pH-values ≥ 10 and are consequently left behind in the aqueous layer (pH ≥ 12) during extraction [2, 20]. 2'-quininone (V) has altered fluorescence properties compared to Q [21] and probably occurs in small quantities in subjects' plasmas compared to Q. Besides, it rapidly enolizes to 2'-OH-Q under the extraction conditions and consequently is retained in the aqueous layer as an anionic species. The non-phenolic hydroxylated metabolites 2-OH-Q and 3-OH-Q (figure 1-III) are co-extracted to a reasonable extent and have fluorescence properties identical to Q [2], 3-OH-Q is formed in appreciable amounts [2, 4]. Therefore it is most likely 3-OH-Q that gives rise to the pronounced peak in the urine chromatograms of the volunteer subject (figure 2b, peak 1). Maybe the minor metabolites 2-OH-Q and the N-oxide contribute to some extent to this peak. At later times of blood-sampling (≥ 4 h after dosage) the same peak is observed in plasma chromatograms too.

In contrast to the 9-OH hydroxy group of quinidine [3], this group in case of Q appears to be a site of glucuronide conjugation. The urine of our pilot pharmacokinetic subject was found to contain 29% more free Q after its treatment with β-glucuronidase than without such a treatment. In accordance with this observation are the findings of Salvadori and co-workers [4], who reported an elevation of the urinary Q recovery of about 20% after a preliminary enzymatic hydrolysis of the urine voidings of their pilot subject.

Four hours was found to be the optimal incubation time of urine samples with β-glucuronidase. Longer incubation times, e.g., during an overnight period, resulted in considerable loss of Q. This loss might be due to bacterial conversion.

Dual plasma peaks have been observed after oral administration of quinidine to human volunteers [22, 23], their occurrence too was attributed to enterohepatic recirculation.

Slight adjustments of the fluorescence excitation and emission wavelengths and maybe of the volume ratio of THF/acetate buffer in the mobile phase render the present assay procedure suitable for the monitoring of e.g., papaverine, propanolol, promethazine, bendrofluazide, quinethazone and quinidine. Injection into the HPLC under the conditions described under 'Apparatus' showed that all these compounds were fluorescence-detected and gave rise to good peak shapes and short retention times.
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SECTION III

EXPERIMENTAL CLINICAL PHARMACOKINETICS

OF NICOTINE, CAFFEINE, AND QUININE
NICOTINE INTAKE BY SMOKERS OF FILTER CIGARETTES.
PART I: CONVENTIONAL FILTER CIGARETTES.

Plasma concentration profiles of nicotine and its main metabolite cotinine were measured over 24 hrs on two separate occasions in a group of 25 habitual smokers, uniformly distributed over 4 current cigarette brands (coded F1 to F4) equipped with conventional (non-ventilated) filter tips. On one of these occasions the smokers were administered a known reference dose of cold-labeled cotinine by mouth.

Pharmacokinetic information calculated from the circadian plasma profiles of nicotine and cotinine derived from smoking and from the single-dose profile of the labeled cotinine was combined with smoking machine data on the relative retention of mainstream smoke nicotine in the cigarette filters in order to estimate the smokers' nicotine intake at mouth-level and at the level of the systemic blood.

The nicotine intake rate at mouth-level amounted to 1.33 ± 0.25 mg/h, corresponding to a nicotine dose of 1.58 ± 0.48 mg, 1.05 ± 0.16 mg, 1.51 ± 0.42 mg, and 1.67 ± 0.48 mg of nicotine delivered per cigarette to the smokers' mouths for the smokers of brands F1 to F4, respectively (mean ± SD).

The bioavailability or fractional uptake of nicotine in the respiratory tract as calculated from the cotinine plasma data amounts to 88 ± 13 %.

It was concluded that for the 4 medium nicotine yield brands studied in this report, the smoking machine standard procedure yields a fairly good indication of the nicotine intake of human smokers.

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¹Department of Pharmacology, Faculties of Medicine and of Natural Sciences and Mathematics, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, and
²Department of Analytical Chemistry, Division of Technology for Society, Netherlands Organization for Applied Scientific Research (T.N.O.), P.O. Box 342, 7300 AH Apeldoorn, The Netherlands.
The amount of nicotine absorbed from the smoke of a cigarette may be deter-
mined by comparing the area under the plasma nicotine vs time curve (AUC) 
during and following cigarette smoking to the AUC observed after intravenous 
administration of a reference dose of nicotine. If cigarette smoking and infu-
sion of (stable isotope-labeled) nicotine are performed simultaneously, the 
rate at which an experimental subject self-administers nicotine by smoking 
cannot be expected to be representative of smoking in everyday life as it is 
affected (slowed) by the intravenous nicotine input.

So, nicotine intake by smoking and by intravenous administration must be 
performed on time-separated occasions, but now short-term intra-individual 
fluctuations in nicotine disposition kinetics might interfere with a clear 
evaluation of the dose of nicotine absorbed from the smoke.

Renal nicotine clearance strongly depends upon urinary pH and flow, 
suggesting variability of the latter quantities (e.g., as a result of food and 
drink intake) as a mechanism of short-term fluctuations of renal nicotine dis-
position rate.

Metabolic nicotine clearance approximates hepatic blood flow and therefore 
is influenced by factors affecting this flow, like physical exercise, posture, 
ingestion of a high protein meal, or the cardiovascular effects of nicotine 
itsclf, the susceptibility to which may vary from one time to another within a 
single individual.

Of course, age and previous exposure to nicotine are examples of factors 
affecting nicotine clearance as well, but these factors are likely to induce 
relatively long-term changes of total nicotine clearance within a given indi-
vidual.

Attempts to minimize intra-individual differences in nicotine disposition 
rate by standardizing one or more of the short-term factors mentioned, e.g., 
by hospitalizing the subjects (meals, posture, physical exercise) or by oral 
administration of ammonium chloride (urinary pH), may be conceived. However, 
as (renal) nicotine disposition rate influences its intake rate, Feyer-
abend et al and Benowitz et al may have standardized nicotine intake of 
their subjects as well, losing relevance of their conclusions for nicotine 
intake in natural smoking circumstances.

In addition, nicotine disposition kinetics is very fast. Monitoring rapid 
increases and decreases of its plasma profile after IV injection or infu-
sion, or after smoking cigarettes requires multiple samples and a strenuous 
sampling scheme, and therefore poses a major interference with a natural smok-
ing behaviour. Furthermore, it is ethically unacceptable to administer the
toxic tobacco alkaloid routinely to large numbers of subjects.

In contrast, the kinetics of the major nicotine metabolite cotinine is
rather slow. Blood sampling may be done at ease, and measured plasma
concentrations are less dependent on the exact times of sampling. Metabolic
conversion of nicotine into cotinine is linear for nicotine input rates up to
at least 30 mg/day, and subsequent elimination of cotinine is independant of
plasma concentrations up to at least 400 μg/l. Cotinine therefore is more
suitable as a marker of nicotine intake in smokers than nicotine itself. Cotinine
displays extremely weak, if any, pharmacological or toxic effects,
and psychological activity. Therefore its administration to volunteer
smokers is free of danger, and its effect on their smoking desire is minimal.
Taking advantage of the rapid and complete absorption of cotinine from the
human GI tract, we determined cotinine clearance in steady smokers by oral
administration of a dose of dideuterated cotinine, smokers meanwhile smoking
in their usual manner. Kinetical evaluation of circadian (24 hr) profiles of
unlabeled cotinine (derived from nicotine) and of cold-labeled cotinine after
ingestion of a known dose enabled us to determine the amount of cotinine gen­
erated from the smoke-absorbed nicotine. By investigating the retention char­
acteristics of nicotine in the filters of the brands studied, we also were
able to obtain information concerning the amount of nicotine delivered to the
mouths of our subjects in the act of smoking, and to obtain a reasonable esti­
mate of the nicotine bioavailability.

The results of this newly designed experiment for 25 smokers are reported.

8.1 MATERIALS AND METHODS

Subjects. Subjects were recruited from the local university student pop­
ulation. Selection criteria included faith to a single brand, inhalation of
the smoke, and a more or less regular consumption pattern, that is, a consump­
tion rate varying only moderately between days, amounting to 20 cigarettes a
day on the average, and a regular within-day spread of cigarettes smoked. Reg­
ularity of consumption was investigated during a week preliminary to the
experiment by having the subjects put a streak for each cigarette smoked on a
paper strip with a time table on it. Subjects were kept unaware of the precise
purpose of their recordings.
### TABLE I. SUBJECT CHARACTERISTICS

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<thead>
<tr>
<th>Brand/ Sex</th>
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<tr>
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<tr>
<td>MEAN</td>
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<tr>
<td>± SD</td>
<td>2 6</td>
<td>1.6</td>
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</table>

**OVERALL MEAN: AGE: 25 yrs, WEIGHT: 63 kg, CIG. CONS.: 21.4 cig/day.**

**OVERALL SD : 4 10 2.1**

BW = body weight, Med = medication, Cig/day = mean number of cigarettes smoked per day in the experimental period, SOC = steroid oral contraceptives.

The selected subjects were 6 males and 19 females, all apparently healthy, between 20 and 40 yrs of age (mean ± SD = 25 ± 4), and having a body weight between 47 and 92 kg (mean ± SD = 63 ± 10). They all gave their written informed consent. All had normal history, baseline hemoglobin and hematocrit. None had any clinical evidence of hepatic, renal, or hematologic disease. The subjects were habitual smokers, who had been smoking their brand for at least 6 months, and engaged in no other nicotine-related practices (cigars, pipes, snuff, chewing tobacco, gum). Their daily cigarette consumption during the experiment was 21.4 ± 2.1 cigarettes.
TABLE II. CHARACTERISTICS OF THE CIGARETTE BRANDS

<table>
<thead>
<tr>
<th>Brand</th>
<th>Tar*</th>
<th>Nicotine*</th>
<th>CO*</th>
<th>Filter length</th>
<th>Overwrap length</th>
<th>Cigarette length</th>
<th>R_nic</th>
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<td>F1</td>
<td>16</td>
<td>1.2</td>
<td>17.5</td>
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<td>26</td>
<td>84</td>
<td>31.9 ± 2.5</td>
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<td>18</td>
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<td>80</td>
<td>35.1 ± 2.1</td>
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<td>84</td>
<td>34.1 ± 2.5</td>
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<td>F4</td>
<td>13</td>
<td>1.0</td>
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<td>21</td>
<td>25</td>
<td>84</td>
<td>31.7 ± 2.4</td>
</tr>
</tbody>
</table>

* Stated by the manufacturers in accordance with ISO 3308 and ISO 3402.

R_nic = relative filter retention of nicotine for machine smoking of the subjects' brands. Listed values are mean ± SD for duplicate groups of 10 cigarettes, machine-smoked at 6 different combinations of puff volume and puff duration.

Eight female smokers took oral contraceptives. No other medications had been received for several weeks by any of the subjects. They all consumed xanthine beverages on a regular basis. Alcohol was consumed in a moderate way (± 10 g/day). Subject characteristics are summarized in Table I. Characteristics of the four cigarette brands smoked are similar (Table II).

Experimental design and sampling. In an experimental period of 16 consecutive days, subjects kept a record of time and number of cigarettes smoked by putting streaks on a time strip. In addition, they pooled the butts saved at each of the 16 days in 55 ml screw-capped containers made of transparent brown polystyrene. At day 4 and day 11 (Tuesdays) subjects attended the laboratory. At about 9.00 a.m. at day 11 (not at day 4) the subjects swallowed a gelatine capsule containing a precisely known amount (± 0.1 mg/kg) of 4',4'-dideutero cotinine (cotinine-D2) with 200 ml of water. In all other respects the experimental protocols at days 4 and 11 were identical.

In the course of these two days, 13 blood samples were taken at 35 min intervals between 9.00 a.m. and 17.00 p.m., allowing a lunch break between 12.30 and 14.00 p.m.. Two further samples were obtained at times between 21.30 and 24.00 p.m., and between 9.00 and 9.30 a.m. next morning. In the morning of days 4, 5, 11, and 12, subjects were not allowed to smoke until the first blood sample was obtained (at ± 9.00 a.m.). Otherwise, no constraints with respect to consumption of cigarettes, meals, alcoholic drinks, and xanthine beverages were imposed on the subjects in order to preserve maximum resem-
ble to their everyday way of smoking. For this same reason, subjects bought their own cigarettes during the experimental period (after termination of the period they received a payment for their participation), and no attempt was made to standardize subjects' urinary flow and pH. Subjects were asked not to behave extraordinarily in any respect in the experimental period.

Blood was centrifuged (1000 g, 10 min) and the plasma was stored at -20°. Samples could be stored for at least 6 months without detectable loss of nicotine, cotinine, and cotinine-D2. Butts of cigarette filters were dark-stored in the polystyrene containers at room temperature. Butts having the tobacco-end of the filter occluded by some residual unburnt tobacco could be stored up to 1 month, nicotine loss in the filter remaining smaller than 10%. As general practice, all butts saved by a subject were analyzed for butt length and nicotine content at the day following the subject's experimental period, butt age then ranging from 1 to 17 days. Nicotine content was measured in 10 selected butts returned by the subject at each day in the experimental period. Criteria for butt selection were a representative average butt length, the absence of lipstick stains as a potential source of compounds interfering with analysis, and the presence of a tuft of unburnt tobacco occluding the filter. Residual tobacco and ashes were removed just prior to analysis.

Nicotine retention in machine-smoked cigarettes. Cigarettes of the brands smoked by our subjects were conditioned to standard conditions of (inter alia) temperature and relative air humidity (ISO 3402, atmosphere A)15, and subsequently machine-smoked with one puff per minute to a butt length equal to the length of the filter + 8 mm (ISO 3308)14. In partial contravention to the standard, puff volume was chosen either 35, 45 or 60 ml, and puff duration either 1 or 2 sec. At each of the six possible combinations of puff volume and duration, duplicate groups of 10 cigarettes of each of the 4 brands were smoked. The particulate matter in the mainstream smoke was trapped on a glass fibre filter ('Cambridge' filter). The amounts of nicotine in the cigarette filter (F) and on the glass fibre filter (D) were determined by GC-NPD. The relative retention of nicotine in the cigarette filter was determined as

\[
R_{nic} = \frac{F}{F + D}
\]  

Drug determinations. Smokers' plasma samples (1 ml) were extracted in duplicate on ethyl silica solid phase extraction columns. Extracts were ana-
alyzed by GC-NPD to determine nicotine and (total) cotinine. Linearity, specificity and sensitivity of the method have been demonstrated for nicotine and cotinine concentrations ranging from 1 - 60 ng/ml and from 5 - 400 ng/ml, respectively. Extracts of plasmas produced at days of cotinine-D2 administration were also analyzed by GC-MS/SIM in order to determine the molar fractions of cotinine (at m/z = 98) and cotinine-D2 (at m/z = 100).

The nicotine amounts retained in cigarette filters and on glass fibre filters were determined by preparing suspensions of the (crushed) filters in methanol and by analyzing filtered suspension aliquots using a GC-NPD method derived from the method used for plasma analysis.

Kinetic calculations. Plasma concentration versus time profiles of cotinine-D2 after oral administration were fitted to a sum of three exponentials:

$$C(t) = \sum_{i}^{2} A_i e^{-t/\tau_i} - e^{-t/\tau_0}$$

From the concentration coefficients ($A_1, A_2$) and the time constants ($\tau_1, \tau_2$), the total area under the plasma concentration versus time curve (AUC) and the total area under the time-concentration vs. time curve (TAUC) were calculated as:

$$\text{AUC} = \sum_{i}^{2} A_i \tau_i - (\sum_{i}^{2} A_i) \tau_0 \quad \text{and:} \quad \text{TAUC} = \sum_{i}^{2} A_i \tau_i^2 - (\sum_{i}^{2} A_i) \tau_0^2$$

Mean residence time (MRT), total clearance (CL), distribution volume ($V_{dss}$), terminal half-life ($t_\frac{1}{2}$), extraction ratio (E), number of recirculations ($N_{rc}$), and mean transit time (MTT) of cotinine-D2 were calculated as:

$$\text{MRT} = \frac{\text{TAUC}}{\text{AUC}} - \tau_0, \quad \text{CL} = \frac{\text{Dose} \cdot h_a}{\text{AUC}}, \quad V_{dss} = \frac{\text{CL} \cdot \text{MRT}}{\text{CL}}, \quad t_\frac{1}{2} = \frac{\tau_2}{\ln 2},
$$

$$E = \frac{\text{CL}}{C.O.}, \quad N_{rc} = \frac{(1-E)}{E}, \quad \text{MTT} = \frac{\text{MRT}}{N_{rc}}$$

Systemic availability ($h_a$) of orally administered cotinine-D2 was assumed to be unity. The cardiac output C.O. was taken 6 l/min = 360 l/hr.

The average nicotine dose D delivered per cigarette to the mouth of a smoker at day 4 or at day 11 of the experimental period was calculated as:

$$D = F \cdot \frac{(1-R_{nic})}{R_{nic}}$$
where \( F \) is the average amount of nicotine found in ten selected butts of the respective subject at the particular day, and \( R_{\text{nic}} \) is the relative retention of nicotine in filters of the smokers' brand. If \( n \) is the number of cigarettes smoked at the day in question, the mean circadian nicotine delivery rate to the smoker's mouth is calculated according to

\[
\dot{D} = n \cdot D / (t_{15} - t_1) \tag{VI}
\]

where the time interval \( (t_{15} - t_1) \) between the 1st and the 15th venipuncture closely approximates 24 hrs.

Plasma concentration-time data \((C_i(t), t_i), \text{ with } i = 1, 2, 15\) of nicotine and unlabeled cotinine were fitted with the aid of the curve fitting FORTRAN programme 'FARMFIT' developed at the Department of Pharmacology in Nijmegen. Both nicotine and cotinine were assumed to have a biexponential transport functions in all subjects. The rapid pulmonary nicotine input in smoking was simulated by considering each cigarette smoked as an intravenously injected dose of nicotine, the slower formation rate of cotinine from nicotine was simulated by the introduction of an exponential absorption-like phase. Subsequently, 'FARMFIT' computed circadian areas \((\text{AUC}_{24})\) under the fitted nicotine and cotinine curves. Pseudo steady-state concentrations \((C_{24}^{\text{nic}})\) of nicotine and cotinine were obtained by division of the respective \(\text{AUC}_{24}^{\text{nic}}\) by the 24 hr time interval \([t_1, t_{15}]\), representing one oscillation period of a steady smoker's circadian nicotine and cotinine plasma concentration profiles.

\[
C_{24}^{\text{nic}} = \frac{\text{AUC}_{24}^{\text{nic}}}{(t_{15} - t_1)} \quad \text{and} \quad C_{24}^{\text{cot}} = \frac{\text{AUC}_{24}^{\text{cot}}}{(t_{15} - t_1)} \tag{VII a/b}
\]

In the characteristic period of a steady smoker's nicotine intake, systemic input rate and output rate for nicotine and cotinine are balanced. The nicotine input (absorption) rate equals its input rate at mouth-level \((\dot{D})\) times its systemic availability \(f_L\). The cotinine input (formation) rate is obtained by multiplication of the nicotine absorption rate by the conversion factor \(f_M\). The disposition rates of both substances equal their respective clearances times their pseudo steady-state concentrations. The plasma mass balance formulas thus read as follows

for nicotine \( D \cdot f_L = CL_{\text{nic}} \cdot C_{24}^{\text{nic}} \tag{VIII a} \)

for cotinine \( D \cdot f_L \cdot f_M = CL_{\text{cot}} \cdot C_{24}^{\text{cot}} \cdot f_{\text{corr}} \tag{VIII b} \)

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The factor \( f_{\text{corr}} \) in eqn VIII b equals 0.921 if the *nicotine* input rate \( \dot{D} \) and the average circadian *cotinine* concentration \( C_{\text{cot}}^{24} \) are expressed on a (m or \( \mu \)) gram basis, rather than on a mole basis, to correct for the different molecular weights of nicotine (162.2) and cotinine (176.2). Rearranging eqns VIII a/b to collocate all measured quantities in the right equation terms, and (hence) all unknowns in the left ones, we obtain

\[
\frac{C_{\text{nic}}}{f_L} = \frac{\dot{D}}{C_{\text{nic}}^{24}} \quad (\text{IX a})
\]

\[
f_L \cdot f_M = \frac{C_{\text{cot}} \cdot C_{\text{cot}}^{24}}{\dot{D}} \quad (\text{IX b})
\]

Although the nicotine systemic availability \( f_L \) and the conversion factor \( f_M \) cannot be solved from eqns IX a/b, the context of the known quantities in these equations confines the theoretical range of values of \( f_L \) and \( f_M \) (from 0 to 1) to a small interval close to unity, as will be shown in the next sections. A fairly accurate estimate of the systemic nicotine intake in our smoking subjects is therefore possible. In particular, after having rationalized that \( f_M \) should approximate 0.75 in the subjects (see 'Discussion'), eqns VIII b and VI were combined to compute the amount of nicotine per cigarette delivered systemically as

\[
D \cdot f_L = (t_{15} - t_{1}) \cdot C_{\text{cot}} \cdot C_{\text{cot}}^{24} \cdot f_{\text{corr}} / (n \cdot 0.75) \quad (\text{X})
\]

8.2 RESULTS

Machinal smoking of brands F1 to F4 revealed a highly linear relationship \((r > 0.98)\) between the amount of nicotine deposited in the cigarette filter and the amount passing through it in the mainstream smoke in the ranges of puff volume and puff duration applied. In these ranges, relative retention of nicotine in the cigarette filter is virtually independent of puff volume and duration and may be regarded in good approximation as a constant for each brand. These constants are included in Table II (right column). About one third of nicotine in the unfiltered smoke offered to the tobacco/filter interface is retained by the filter for all four brands studied. The complementary two thirds pass through it and are delivered to the smoker's mouth. A similar relative retention (37%) was observed by Hopkins et al. for another filter brand.
FIGURE 1 Circadian nicotine (o), cotinine (*) and cotinine-D2 (x) plasma concentration profiles in subject nr 24 at day 4 (upper panel) and day 11 (lower panel) of the experimental period, which were the two days of all subjects' laboratory attendance. Each bottom arrow denotes the (time of) smoking of a single cigarette by this subject.
TABLE III. PHARMACOKINETIC PARAMETERS OF COTININE(-D2) IN OUR SUBJECTS

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<tr>
<th>MAT</th>
<th>MRT</th>
<th>CLcot</th>
<th>Vdss</th>
<th>t1/2</th>
<th>E</th>
<th>Hrc</th>
<th>MTT</th>
</tr>
</thead>
<tbody>
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<td>hr</td>
<td>hr</td>
<td>l/hr</td>
<td>t</td>
<td>hr</td>
<td>o/oo</td>
<td>min</td>
<td></td>
</tr>
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<td>------</td>
<td>------</td>
<td>------</td>
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<tr>
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<tr>
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<td>11.0</td>
<td>1.8</td>
<td>1.8</td>
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</tr>
</tbody>
</table>

Kinetic parameters were calculated from the plasma profile following oral administration of ± 0.1 mg/kg of dideuterated cotinine in a gelatine capsule.

The nicotine dose \( D \) reaching the mouths of our subjects per cigarette smoked therefore is about twice the average filter nicotine content of subject-smoked cigarettes. At the days of laboratory attendance it averages 1.45 ± 0.28 mg for all subjects, and is similar (1.5 - 1.7 mg) for brands F1, F3, and F4 (see Table IV left-hand column, and figure 2). The nicotine yield of F2 is lower than that of the other brands (but not significantly so) and is even slightly lower than its standard smoking machine yield (Table II, figure 2). Nicotine delivery per cigarette \( (D) \) and per hour \( (\dot{D}) \) are similar (Table IV, two left-most columns) because subjects of all subjects smoked about one cigarette per hour \( (= 24/day; \) see Table I).

In figure 1 circadian nicotine and cotinine plasma concentration profiles of a representative subject are shown for the two days of the subject's laboratory attendance: day 4 (upper panel) and day 11 (lower panel). The nicotine and cotinine curves during these days are rather congruent, and as a consequence, pseudo steady state concentrations of these compounds are quite similar at days 4 and 11. Concentrations of nicotine and cotinine are lowest in the morning and start rising when smoking is initiated. After about 5 cigarettes, in this subjects as in all subjects a nicotine plateau level is attained. Cotinine concentrations keep rising until late in the afternoon or evening. During the night, both curves return to approximately their pre-smoking morning values and thereby close the circadian cycle.

In the lower panel the concentration profile of orally administered cotinine-D2 is displayed. Cotinine is absorbed rapidly from the GI tract, mean absorption time amounting to 1 hr (Table III). Its volume of distribution indicates a slight extent of tissue cumulation. Cotinine disposition is rather slow as indicated by its mean residence time of 14 hrs, its terminal half-life of 12 hrs, and its low total clearance of \( 3\frac{1}{2} \) l/hr. The extraction ratio reveals 1% of cotinine molecules to be eliminated during each body transit of
6.3 min. On the average, a cotinine molecule may complete some 100 circulations before being disposed of.

These pharmacokinetic parameters largely confirm those reported by De Schepper et al., Benowitz et al., and Kyerematen et al. In particular, the values of total cotinine clearance encountered in the present study are less than those observed in intravenous studies conducted by De Schepper et al. \((3.66 \pm 0.57 \text{ l/hr})\) and Benowitz et al. \((4.33 \pm 0.79 \text{ l/hr})\), implicating complete systemic availability of cotinine after ingestion as a gelatine capsule by our subjects. Pharmacokinetic parameters of cotinine did not differ between men and women, nor between the subject populations of the 4 brands.

In Table IV, measured and computed parameters of nicotine and cotinine are listed for all subjects at both days of laboratory attendance. Parameters have been averaged for the smokers' population of each brand, as well as for all subjects. Nicotine and cotinine pseudo steady-state concentrations \((C^{24})\) are intra-individually very reproducible, but assume a wide range of values between subjects. Cotinine levels are more than tenfold higher than nicotine levels; the reverse is true for the clearances of these compounds.

Assuming the nicotine availability \(f_L\) to be close to unity, the quotient \(CL_{nic}/f_L\) may be regarded as a slight overestimation of the nicotine clearance \(CL_{nic}'\), which thus is seen to amount to 50 - 60 l/hr and to approximate the hepatic blood flow.

Footnotes ad Table IV
* Brand mean ± std. deviation, ** overall mean ± standard deviation.
@ For each subject, the upper row of figures refers to the 1\(^{st}\) day, and the lower row to the 2\(^{nd}\) day of laboratory attendance (days 4 and 11 of the experimental period, respectively).

\[ \begin{align*}
D & \quad \text{mean nicotine input at mouth-level per cigarette} \\
\dot{D} & \quad \text{mean nicotine input at mouth-level per hour} \\
C^{24}_{nic} & \quad \text{mean circadian nicotine plasma concentration} \\
C^{24}_{cot} & \quad \text{mean circadian cotinine plasma concentration} \\
CL_{nic}/f_L & \quad \text{total nicotine clearance divided by its bioavailability} \\
CL_{cot} & \quad \text{total cotinine plasma clearance} \\
EL_{cot} & \quad \text{mean circadian cotinine elimination rate} = CL_{cot} \cdot C^{24}_{cot} \\
f_L \cdot f_M & \quad \text{nicotine availability} \cdot \text{fraction converted into cotinine} \\
D*f_L & \quad \text{mean systemic (blood-level) nicotine input per cigarette, } f_L \text{ is calculated from the fraction product } f_L \cdot f_M \text{ for } f_M = 0.75
\end{align*} \]
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<th>( D ) (mg/cig)</th>
<th>( \bar{d} ) (mg/hr)</th>
<th>( C_{\text{nic}}^{24} ) (( \mu \text{g/l} ))</th>
<th>( CL_{\text{nic}}/f_L ) (l/hr)</th>
<th>( C_{\text{cot}}^{24} ) (( \mu \text{g/l} ))</th>
<th>( CL_{\text{cot}} ) (l/hr)</th>
<th>( EL_{\text{cot}} ) (mg/hr)</th>
<th>( f_L \cdot f_M )</th>
<th>( D \cdot f_L ) (mg/cig)</th>
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TABLE IV (cont’d). NICOTINE INPUT AT MOUTH- AND AT BLOOD-LEVEL, CIRCADIAN PLASMA LEVELS AND CLEARANCES OF NICOTINE AND COTININE, ELIMINATION RATE OF COTININE, AND SYSTEMIC AVAILABILITY OF NICOTINE TIMES ITS FRACTIONAL CONVERSION INTO COTININE IN THE SUBJECTS (see notes)

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<th>C24 cot</th>
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<th>EL cot</th>
<th>fL*fM</th>
<th>D*fL</th>
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<td>mg/cig</td>
<td>mg/hr</td>
<td>µg/l</td>
<td>l/hr</td>
<td>µg/l</td>
<td>l/hr</td>
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140
Systemic availability of inhaled nicotine (f_L) and its extent of conversion into cotinine (f_M) must both be high, since nicotine delivery rate (D) and cotinine disposition rate (EL_COT) diverge less than a factor two (Table IV). The fraction product f_L*f_M calculated from these mass transfer rates averages 0.66 ± 0.10 in our subjects, and is similar for the populations smoking the 4 brands. However, it was noticed that the sequences of magnitude of f_L*f_M and the dose of nicotine delivered per cigarette at mouth-level run antiparallel for the 4 brands, suggesting (a smoking technique aimed at) higher nicotine availability in subjects with decreasing mouth-level delivery of their brand.

The value of the product f_L*f_M draws the lower line for the value of both fractions at 0.66 on the average. On the basis of few literature information (see: 'Discussion'), we estimated f_L and f_M to average 0.88 and 0.75, respectively, in our subjects. Systemic nicotine delivery per cigarette (D*f_L) was calculated for a value of f_L calculated on basis of f_M = 0.75, and is included in Table IV. Mouth-level and systemic nicotine delivery for the smokers of all brands have been visualized in figure 2.

8.3 DISCUSSION

The approximate proportionality between the amount of nicotine (and 'tar') retained by a cigarette filter and the amount passing through has been recognized previously. In agreement with this previous work, the relative retention of nicotine in the cigarette filter was found to depend on smoking parameters, and in particular with the quotient of puff volume and puff duration, which is the smoke flow through the filter during a puff. Relative nicotine retention decreased slightly with increasing smoke flow, suggesting interference of short smoke filter transit times with aerosol deposition in the filter. In the range of smoke flows applied (17.5 - 60 m/sec), relative nicotine retention in the filters of the 4 brands studied was of sufficient constance to be useful as a tool for calculation of mouth-level nicotine exposure of our subjects.

Puff volume and puff duration in machine smoking were chosen so as to cover the range of values frequently employed by smokers, according to smoking topography studies. Available data indicate that these and other smoking topography parameters are subject to considerable variability from puff to puff, cigarette to cigarette, and from one smoker to another.
FIGURE 2 Nicotine deliveries of the four cigarette brands studied. $D^*$ = nicotine yield by standard smoking-machine procedure, $D^{**}$ and $D^{***}_{fl}$ = nicotine intake at mouth-level and at blood-level (systemic level), respectively, of the subject populations of the four cigarette brands. Numbers given are mg of nicotine per cigarette. Mean values and standard deviations have been indicated numerically and graphically.

Brand nicotine deliveries to our subjects did not differ significantly from each other, nor from the nicotine yields as determined by the standard machine smoking test of each brand, the result of which is mentioned on the packet (except for F4 delivery at mouth- and at blood-level of this brand were significantly higher than its machinal delivery ($P < 0.02$ by two-tailed t-test)).

In view of these fluctuations we averaged relative nicotine retentions over the various circumstances of puff volume and puff duration at which each brand was machine-smoked, and used the resultant overall retention for computation of mouth-level deliveries 'in vivo'.
Reliability of this approach was further enhanced by analyzing nicotine in filters of subject-smoked cigarettes taking 10 butts per day and per subject, the random check thus amounted to nearly half the whole amount of butts! Furthermore, nicotine loss was investigated as a function of time and storage mode, the best storage mode and an acceptable storage time were employed. We query the suitability of the storage procedures reported by Forbes et al.\textsuperscript{17} (butt freezing) and by Hopkins et al.\textsuperscript{16} (removal of residual tobacco before storage). In our hands both procedures yielded inferior results.

The validity of the present approach is supported by the amounts of nicotine found in filters of subject-smoked cigarettes, which were nearly all in the range obtained when machinally smoking the corresponding brand under the conditions specified. In addition, the retention data derived by machine-smoking of the 4 brands are consistent with the information obtained by plasma concentration measurements of nicotine and cotinine. Combination of these 'in vitro' and 'in vivo' data, respectively, did not result in abnormally high or low nicotine clearances, fraction products $f_L f_M \geq 1$, or other clear-cut artifacts, not even at one of the 50 experimental days for which 'in vitro' and 'in vivo' data were combined.

Previous studies have shown that time and number of cigarettes smoked can be self-monitored reliably by smokers, the main condition required being that no attempts are made to change their smoking behaviour.\textsuperscript{21,22} Our subjects were encouraged to smoke in their usual way, and were left in their natural environment, except of course at the days of laboratory attendance. Paper strips for recording smoking times were given dimensions that suited them to be stored in the cigarette packets, in order to let 'smoking accounts' interfere minimally with smoking behaviour. Some subjects who counted streaks themselves at the end of each day were amazed that they smoked 'so much'. Smoking rates and mouth-level nicotine delivery of these subjects did not decrease in the course of the experimental period, nor did those of the other subjects.

For each subject, we investigated whether the following quantities exhibited significant differences at each of the days of laboratory attendance, and at the 14 days of non-attendance: butt length, number of cigarettes smoked, amount of nicotine retained in the filters, and the product of the latter two quantities. In all cases, differences were far from significant. However, minimal and maximum amount of filter nicotine as daily analyzed in (and averaged for) 10 filters, invariably diverged by a factor $1\frac{1}{2} - 2$ for all subjects for days of non-attendance. Intra-individual smoking technique
appears to fluctuate not only from puff to puff and from cigarette to cigarette, but also rather long-term, from day to day. Possible differences in filter nicotine content for days of laboratory attendance on the one hand and for those of non-attendance on the other could be camouflaged by the (greater) differences existing between days of non-attendance as a result of other-than-experimental factors affecting smoking behaviour. For what it is worth, we conclude that the smoking behaviour of our subjects was not, or at least not markedly, affected by the experimental protocol.

Total nicotine clearance averaged $f_L = (60.0 \pm 17.8) \ t/hr$ in our subjects (Table IV), whose urinary pH remained uncontrolled in the experiments. For high systemic availability of inhaled nicotine ($f_L = 1$), this finding compares well to those observed after iv nicotine administration in subjects with alkaline urine (pH > 7) by Rosenberg et al. $f_L = (58.0 \pm 5.3) \ t/hr$ in 6 men after a single injection, $46.7 \pm 3.0 \ t/hr$ in 5 men for repetitive injections), in subjects with acidified urine (pH < 5) by Rosenberg et al. $f_L = (78.5 \pm 10.1) \ t/hr$, 5 men, single inj., $61.6 \pm 5.8 \ t/hr$, 5 men, repetitive inj.), by Benowitz et al. $f_L = (77.5 \pm 17.6) \ t/hr$, 5 men), and by Feyerabend et al. $f_L = (54.9 \pm 8.8) \ t/hr$, 5 men), or in subjects with uncontrolled, physiological urinary pH by Benowitz et al. $f_L = (123 \pm 0.30) \ t/hr/kg$ in 13 men, $94.0 \pm 0.28 \ t/hr/kg$ in 9 women.

Scarce literature reports estimate nicotine availability in habitually inhaling cigarette smokers to assume a value $0.75 - 1.00^{23}, > 0.90^{24},$ and $0.82 - 0.92^{25}$. Taking $f_L = 0.88$ in the average subject equates mean total nicotine clearance to an acceptable $52.8 \ t/hr$, at the same time equating mean $f_M$ to the plausible value of 0.75. A high fractional conversion of nicotine to cotinine was expected beforehand on the basis of the relative small abundance of the other primary nicotine metabolites (cis and trans) nicotine-1'-N-oxide, normanor-nicotine, and the isomethylnicotinium ion, in smokers' body fluids. Gori et al. $f_N = 26$ and Cohen & Roe. $f_N = 27$ have speculated $f_M$ to approximate 0.70, which value for our subjects appears rather conservative regarding the close proximity of the lower deadline for $f_M$ obtained in the present study ($= 0.66$).

Returning to the nicotine clearance, then, $52.8 \ t/hr$ is in the lower range of literature values. However, literature data on nicotine clearance were obtained in experiments with men $^{1,2,3}$ performed during daytime. $^{1,2,3,5}$ As women (n=19) outnumber men (n=6, Table I), in this study and eliminate nicotine at a significantly slower rate than men, and as nicotine recirculation by reabsorption from the urine bladder may manifest itself especially during nighttime when bladder emptying is less frequent than during daytime, a low clearance value in our subjects may be explained. Nicotine reabsorption from
the bladder was indicated by the nicotine concentrations in the presmoking 9:00 a.m. plasma samples. With few exceptions, these concentrations usually exceeded predictions on the basis of previous day plateau concentrations combined with a conservative estimate of nicotine elimination half-life of 2 hrs. The discrepancy could not be attributed to extraneous nicotine introduced into the sample during storage or analysis since sources of contamination were under control. A sex difference in the quotient CL\textsubscript{nic}/f\textsubscript{L} was not observed in our subjects, presumably because of the smallness of our male subject population, and because random values of f\textsubscript{L} may camouflage significant differences in nicotine clearance.

Within-subject variability of nicotine clearance (divided by f\textsubscript{L}) at the two days of laboratory attendance, separated by 6 days, ranged from 0.4 to 20.6%, largely confirming figures of Benowitz et al.\textsuperscript{2} (0.3 - 6.4%, 3 days interspacing). Between-subject variability of nicotine clearance (*f\textsubscript{L}^{-1}) was as great as threefold. Inter-individual differences in nicotine disposition rate of this magnitude are encountered commonly\textsuperscript{2,16,28}, even in studies where renal nicotine clearance (urinary pH) was standardized\textsuperscript{5}. Consequently we agree with Benowitz et al\textsuperscript{2} that the accuracy of estimates of a smoker's nicotine intake from the area under his/her circadian plasma nicotine concentration curve is greatly improved if the smoker's individual nicotine clearance is known from measurement on a separate occasion (e.g., the previous or subsequent day).

Our approach of nicotine intake estimation involving circadian AUC measurement of nicotine and cotinine was similar to the strategy of Benowitz et al.\textsuperscript{2,29,30,34} In contrast to these experiments, our subjects were not hospitalized and hence it was practically unfeasible to obtain more than one blood sample in the evening/night from them. Using the trapezoidal rule for AUC calculation\textsuperscript{2,32}, would in case of our data result in a substantial overestimation of (especially nicotine) AUC in the nocturnal phase of the experiment (see fig 1). Therefore we computed AUC by fitting procedures using biexponentials.

Pseudo steady-state concentrations of nicotine and cotinine are intr-individually very similar at the two days of laboratory attendance, presumably resulting from smokers' strive to attain a nicotine level within a relatively narrow range ('index of indulgence'), in which nicotine dynamics are appreciated best\textsuperscript{33}. Similarity of the cotinine levels then follows from the proportionality between nicotine concentration and cotinine formation rate\textsuperscript{6}, and from within-subject constancy of cotinine disposition kinetics. Inter-individually, plasma concentrations differ widely as a result of differences...
in nicotine intake and disposition rate, extent of conversion to cotinine, and cotinine disposition rate. The time course of the profiles show a nicotine plateau reached by noon and a cotinine plateau reached in the late afternoon/evening, designating the latter phase of day as most suitable for obtaining a characteristic indication of a smoker's nicotine and cotinine exposure if the number of samples is restricted to just one, as is practice in epidemiological investigations. Our findings thus support the design of the Gori and Lynch study.

The amount of nicotine delivered systemically per cigarette to the smokers on the average comes to $1.34 \pm 0.34$, $0.96 \pm 0.20$, $1.27 \pm 0.28$, and $1.31 \pm 0.22$ for brands F1 to F4, respectively (Table IV). These amounts differ by $+12, -13, +15,$ and $+31$% from the nicotine labels printed on the packets (Table II). In view of the great differences that may exist between the standard machine-smoking procedure and individual human smoking patterns, it is remarkable to observe the relative proximity of the intake figures predicted by the smoking machine and observed for the small subject populations of each of the 4 (medium nicotine-yield) brands.

The results of a similarly designed experiment for subjects smoking cigarette brands equipped with ventilated filters will be presented in a forthcoming report.

ACKNOWLEDGEMENTS

The authors sincerely thank all subjects having participated in the presently described investigation for their excellent cooperation in experiment performance. The W.A.R.G. (Scientific Advisory Council for Smoking and Health) is gratefully acknowledged for the continuous interest and the financial support provided.
8.4 REFERENCES

11. Teeuwen HWA (1988) Clinical pharmacokinetics of nicotine, caffeine, and quinine. A systems dynamics approach. (a) 'Estimation of the nicotine content of cigarette filters and glass fibre ("Cambridge") filters' (chapter 3), (b) 'Simultaneous estimation of nicotine and cotinine levels in biological fluids using high-resolution capillary-column gas chromatography combined with solid phase extraction work-up' (chapter 2), (c) Capillary gaschromatographic - mass spectrometric - selective ion monitoring method for measuring relative levels of cotinine and orally administered cotinine-D2 in smokers' blood plasmas (chapter 4), (d) 'Carbon monoxide intake by smokers of conventional and ventilated filter cigarettes' (chapter 10). Ph.D. Thesis, University of Nijmegen, Nijmegen, The Netherlands.


15. ISO 3402. Tobacco and tobacco products. Atmospheres for conditioning and testing (19??).


Plasma concentration profiles of nicotine and its main metabolite cotinine were measured over 24 hrs on two separate occasions in a group of 10 habitual smokers, distributed over two current ventilated cigarette brands coded VI and V2. On one of these occasions the smokers were administered a peroral reference dose of cold-labeled cotinine.

Pharmacokinetic information calculated from the circadian plasma profiles of nicotine and cotinine derived from smoking and from the single-dose profile of the labeled cotinine was combined with smoking machine data on the relative retention of mainstream smoke nicotine in the cigarette filters in order to estimate the smokers' nicotine intake at mouth-level and at the level of the systemic blood.

The nicotine intake rate at mouth-level amounted to 1.68 ± 0.60 mg/hr for the 6 subjects smoking brand VI and to 0.63 ± 0.25 mg/hr for the 4 subjects smoking brand V2. These input rates correspond to a per cigarette nicotine intake at mouth-level of 1.67 ± 0.46 mg, and 0.73 ± 0.25 mg of nicotine for the smokers of VI and V2, respectively (mean ± SD).

The bioavailability or fractional uptake of nicotine in the respiratory tract as calculated from the cotinine plasma data amounts to 91 ± 13 \%.

It was deduced that the two ultra-low cigarette brands studied in this report yielded substantially more nicotine to our habitual smokers of these brands than the imprint on the packet predicts.

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In a previous article, we reported the nicotine intake of habitual smokers of medium nicotine yield cigarettes equipped with conventional (i.e., non-ventilated) filters. These intake figures approximated the machine-determined nicotine labels on the cigarette packet rather well. On the basis of compensatory smoking behaviour, which is well-documented in literature, it is expected that for both high and (ultra) low nicotine yield cigarettes, machine and human intake figures would deviate more from each other.

Using the same approach of combining 'in vitro' data relative nicotine retention in cigarette filters, and 'in vivo' data circadian plasma concentrations of nicotine, its main metabolite cotinine and the plasma concentration profile of an orally administered cold isotope-labeled cotinine analogue (4',4'-dideutero cotinine, cotinine-D2), we subsequently investigated the nicotine intake in 10 habitual smokers of two currently marketed brands of ventilated filter cigarettes.

Philosophy and design of the experiment have been outlined previously. The intake figures were compared to the machine nicotine yield of the brands and to the intakes of 25 smokers of conventional cigarette brands reported before.

9.1 MATERIALS AND METHODS

Subjects. Subjects were recruited from the local university student population. Selection criteria included faith to a single brand, inhalation of the smoke, and a more or less regular consumption pattern, that is, a consumption rate varying only moderately between days, amounting to ≥ 20 cigarettes a day on the average, and a regular within-day spread of cigarettes smoked. Regularity of consumption was investigated during a week preliminary to the experiment by having the subjects put a streak for each cigarette smoked on a paper strip with a time table on it. Subjects were not informed of the purpose of their recordings.

The selected subjects were 2 males and 8 females, all apparently healthy, between 19 and 44 yrs of age (mean ± SD = 29 ± 9), and having a body weight between 51 and 76 kg (mean ± SD = 63 ± 9). They all gave their written informed consent. All had normal history, baseline hemoglobin and hematocrit. None had any clinical evidence of hepatic, renal, or hematocrit disease. The
### TABLE I. SUBJECT CHARACTERISTICS

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</table>

**MEAN**

| 34 61 23.8 | 23 65 19.6 |

| ± SD | 8 9 4.3 | 4 9 1.5 |

OVERALL MEAN: AGE: 29 yrs, WEIGHT: 63 kg, CIG. CONS.: 22.1 cig/day.

OVERALL SD: 9 4.0

**BW** = body weight, **Med** = medication, **Cig/day** = mean number of cigarettes smoked per day in the experimental period, **Lo** = user of Lomusol® and Lomeda® (cromoglycate), **SOC** = steroid oral contraceptives.

### TABLE II. CHARACTERISTICS OF THE CIGARETTE BRANDS

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<td>32</td>
<td>84</td>
<td>64 ± 1</td>
<td>3 ± 1</td>
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</table>

* Stated by the manufacturer in accordance with ISO 3308 and ISO 3402.
* VR_{nom} and VR_{min}, nominal and minimal ventilation rate (this chapter).

Subjects were habitual smokers, who had been smoking their brand for at least 6 months. Their daily cigarette consumption during the experiment was 22.1 ± 4.0 cigarettes.

Three female smokers used steroid oral contraceptives, and another female subject took cromoglycate. All subjects consumed xanthine beverages on a regular basis. Alcohol was consumed moderately (± 10 g/day). Subject char-
acteristics are summarized in Table I Data on the two ventilated cigarette brands are listed in Table II.

Experimental design and sampling. Venipuncture schedule, administration of cotinine-D2, collection, storage, and selection of subjects' cigarette butts, and plasma and butt analysis for nicotine and related compounds were performed identically to a previous study la,b,c,d.

Nicotine retention in machine-smoked cigarettes. Overwraps of cigarettes of both brands were treated with a mixture of polyvinyl acetate resin and starch-paste in order to block ventilation holes to a variable degree. Another aliquot of cigarettes remained untreated to preserve the nominal (maximum) ventilation rate. After drying of the lime, cigarettes were placed one at a time in a specially designed holder. While drawing in air at the mouth end of the filter at a flow rate of 17.5 ml/s, the air flow entering the tobacco rod of the cigarette was measured (ml/s) Thus, the ventilation rate (VR) of each individual cigarette was computed as

$$\text{VR} = \left( \frac{17.5 - v'}{17.5} \right) \times 100\%$$

Untreated cigarettes of both brands showed nominal ventilation rate, VR_{nom} (Table II), and constituted one ventilation class. Treated cigarettes were selected to constitute three further ventilation classes, having ventilation rates of about 2/3*VR_{nom}, 1/3*VR_{nom}, and VR_{min} respectively. The VR_{min} class consisted of cigarettes whose perforations had all been blocked by the lime treatment. Due to filter paper porosity, their ventilation rate actually did not equal zero, but rather ranged from 2 to 7 % (Table II). Each class encompassed 140 cigarettes (a total of 560 cigarettes per brand). Absolute standard deviations of VR of the cigarettes in each class were about 1.0 %.

Cigarettes of each ventilation class and brand were conditioned for 48 hrs to standard conditions of temperature and relative air humidity (ISO 3402, atmosphere A)², and subsequently machine-smoked with one puff per minute to a butt length equal to the length of the overwrap + 3 mm (ISO 3308)³, using a Filtrona SM 302 smoking machine equipped with 8 smoking channels. In partial contravention to the conditions stipulated in ISO 3308, puff volume was chosen either 35, 45, 60, or 75 ml, and puff duration either 1 or 2 sec. At seven of the eight possible combinations of puff volume and duration (45 ml/2 sec was arbitrarily omitted), duplicate groups of 10 cigarettes of each ventilation class and brand were smoked. The particulate matter in the mainstream smoke
was trapped on a glass fibre filter (a so-called 'Cambridge' filter). The amounts of nicotine in the cigarette filter (F) and on the glass fibre filter (D) were determined by GC-NPD. For both brands, plots of D versus F were constructed and linear least squares regression parameters were calculated. In addition, correlation coefficients and 95% confidence prediction limits of D for given F were computed.

The average amount of nicotine delivered per cigarette to the mouth of smoking subjects at a particular day in the experimental period (comprising 16 days) was estimated by substituting the average nicotine content (F) of 10 selected representative butts (smoked by the subject at the particular day) into the regression line of the appropriate brand.

Drug determinations. Smokers' plasma samples were analyzed for nicotine, cotinine, and, whenever appropriate, for cotinine-D2 according to specific and sensitive methods described elsewhere\textsuperscript{1b,c}. Nicotine amounts retained in cigarette filters or trapped on glass fibre filters were determined by preparing suspensions of the filters in methanol and by analyzing filtered suspension aliquots using an assay derived from the method used for plasma analysis\textsuperscript{1d}.

Kinetic calculations. Pharmacokinetic and input rate calculations were done as described in the previous chapter\textsuperscript{1a}.

9.2 RESULTS

A grossly linear relationship was observed between the amount of nicotine retained in the cigarette filter and the amount passing through it in the mainstream smoke during machine smoking of brands V1 and V2 (figure 1). However, in contrast to conventional cigarettes\textsuperscript{1a}, the relative retention of nicotine in the ventilated filters of V1 and V2 was not a constant within narrow boundaries, but rather appeared to depend on puff volume, puff duration and ventilation rate. These fluctuations of the relative retention resulted in the ribbon-like appearance of the data points shown in figure 1. Correlation coefficients of regression lines were substantially lower than those calculated for conventional cigarettes, resulting in rather large prediction limits of D computed according to the regression lines for given F.
Averaged over all machine-smoking parameters employed, $R_{\text{nic}}$ amounted to 52% for brand VI and to 68% for brand V2, both values being higher than the 31-35% encountered for the conventional brands F1-F4 studied previously\textsuperscript{1a}.

From figure 1 it may be discerned that for high puff volumes and low ventilation rates the amount of nicotine in the mainstream smoke can mount up to as much as 10 times the nominal value of the label on the cigarette packet of V1 and V2 (Table II). The nicotine dose $D$ delivered at mouth-level to the volunteer smokers at days of laboratory attendance averages $1.67 \pm 0.46 \text{ mg/cig}$ for the subjects smoking V1 and to $0.73 \pm 0.25 \text{ mg/cig}$ for those smoking V2 (Table IV). These amounts differ significantly from the nominal machine yields of these brands (V1 $P < 0.0005$, V2 $P < 0.01$) and from each other ($P < 0.01$), strongly suggesting a smoking technique characterized by a less-than-nominal ventilation rate, larger puff volumes, and/or higher puff frequencies to have been employed by our human smokers as compared to the parameters used in the standard machine-smoking procedure. Mouth-level nicotine yield of brand V1 is statistically indistinguishable from those of the 4 conventional filter brands (F1 to F4) studied previously\textsuperscript{1a}, whereas V2 per cigarette yields less nicotine than F1 ($P < 0.01$), F2 (NS), F3 ($P < 0.05$) and F4 ($P < 0.01$) at mouth-level.

Nicotine delivery per cigarette ($D$) and per hour ($\hat{D}$) for most smokers are similar (Table IV) as most of them smoked about a cigarette each hour when calculated over a circadian period ($= 24 \text{ cig/day}$).

In figure 2 circadian nicotine and cotinine plasma concentration vs. time profiles of a subject smoking brand V2 are shown for the two days of laboratory attendance: day 4 (upper panel) and day 11 (lower panel). In the lower panel the profile of cotinine-D2 is shown as well.

---

**FIGURE 1. Relationship between the amount of nicotine present in the mainstream smoke ($D$) and the amount retained in the cigarette filter ($F$) in machine smoking of brands VI and V2.** Smoking parameters: puff volume/puff duration $\Diamond = 35 \text{ ml/2 s}$, $\circ = 35 \text{ ml/1 s}$, $\square = 45 \text{ ml/1 s}$, $\vartriangle = 45 \text{ ml/1 s}$, $\bigcirc = 60 \text{ ml/1 s}$, $\bigtriangledown = 60 \text{ ml/2 s}$, $\blacklozenge = 75 \text{ ml/1 s}$, $\blacktriangle = 75 \text{ ml/1 s}$. Open symbols = nominal ventilation rate ($VR_{\text{nom}}$), symbols blackened less than half $2/3*VR_{\text{nom}}$ symbols blackened more than half. $1/3*VR_{\text{nom}}$ totally blackened symbols: minimal ventilation rate ($VR_{\text{min}}$). Regression lines: $\forall 1. D = 1.0789*F - 0.1778$, $n = 56$, $r = 0.816$ $\forall 2. D = 0.5433*F - 0.0956$, $n = 56$, $r = 0.873$. 95% Confidence prediction intervals of $D$ for given $F$ have been indicated.
FIGURE 2. Circadian nicotine (o), cotinine (*), and cotinine-D2 (▼) plasma concentration profiles in subject nr. 32 at day 4 (upper panel) and at day 11 (lower panel) of the experimental period, which were the two days of all subjects' laboratory attendance. Arrows denote the (time of) smoking of a single cigarette by this subject. (Cf. fig. 1, chapter 8).
TABLE III  PHARMACOKINETIC PARAMETERS OF COTININE(-D2) IN THE SUBJECTS

<table>
<thead>
<tr>
<th>MAT</th>
<th>MRT</th>
<th>CLcot</th>
<th>Vdss</th>
<th>t1/2</th>
<th>E</th>
<th>Nre</th>
<th>MTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>hr</td>
<td>l/hr</td>
<td>l</td>
<td>hr</td>
<td>o/oo</td>
<td>min</td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>0.81</td>
<td>15.2</td>
<td>3.56</td>
<td>53.9</td>
<td>12.4</td>
<td>9.9</td>
<td>101</td>
</tr>
<tr>
<td>± SD</td>
<td>0.30</td>
<td>2.4</td>
<td>0.43</td>
<td>10.2</td>
<td>1.8</td>
<td>1.2</td>
<td>13</td>
</tr>
</tbody>
</table>

Kinetic parameters were calculated from the plasma profile following oral administration of ± 0.1 mg/kg of dideuterated cotinine in a gelatine capsule.

Pharmacokinetic parameters of cotinine as calculated from the plasma profiles following oral administration of cotinine-D2 in a gelatine capsule (Table III) agreed with those observed for the smokers of conventional filter cigarettes and those observed by Benowitz et al. 6, De Schepper et al. 7, and Kyerematen et al. 8. Combining the present data with those of the conventional filter cigarette study, 1a we found none of the kinetic cotinine parameters to be significantly different between the smokers' populations of the six brands (VI, V2, F1-F4), between sexes, or between female subjects taking steroid oral contraceptives (representing about 40% of the women smokers both studies) and those not taking SOC.

Circadian plasma levels (C24h) of nicotine and cotinine average 28.4 and 326 ng/ml for smokers of VI, and 10.4 and 139 ng/ml for those of V2, respectively (Table IV). The levels of the smokers of VI are slightly higher, those of the V2-smokers substantially lower than those observed in smokers of the conventional cigarettes F1-F4. The differences are entirely attributable to proportional differences in nicotine intake rate at mouth-level, since systemic availability of smoke-inhaled nicotine (fL), nicotine total clearance (CLnic), fractional conversion of systemic nicotine into cotinine (fH), and cotinine total clearance (CLcot) appear to be similar for the smokers' populations of all brands (Table IV, cf. Table IV in chapter 8). However, note that the fraction product fL*fH of the lowest-delivery brand V2 (= 0.71) is higher than observed for any other brand, confirming our earlier suggestion of (a smoking technique aimed at) higher nicotine availability in smokers of low nicotine brands.

Scarce literature information indicates smoke-inhaled nicotine availability (fL) to approximate unity and the fractional conversion of systemic nicotine into cotinine (fH) to be around 0.7. Like in the conventional filter cigarette study, in this study fH was taken 0.75 as a mean value for our subjects. Fractional uptake of nicotine based on the product...
(Table IV) for $f_M = 0.75$ can be shown to average $f_L = 0.88 \pm 0.15$ in smokers of V1 and $f_L = 0.95 \pm 0.12$ in smokers of V2. Individual values of $f_L$ were used to compute the nicotine dose $D*f_L$ delivered systemically (absorbed into the pulmonary blood). These values have been included in Table IV (right-hand column) and are also visualized in figure 3 for the smokers' populations of V1 and V2.

9.3 DISCUSSION

In case of ventilated filters, linear velocity and flow rate of the smoke during a puff are not merely a function of puff volume, puff duration, and cigarette diameter, but rather are influenced by vent air and hence by ventilation rate, as indeed was experimentally observed by us and by others. The higher the ventilation rate, the more air is drawn in during a puff, reducing flow and linear velocity of the smoke. As a result, deposition of particulate matter in unburnt tobacco and cigarette filter will be promoted. In the present study, cigarettes of V1 and V2 were machine-smoked with ventilation rates varying from 3-6% to 64-81% (Table II), effectuating substantial elevations of aerosol transit time in the filters. This phenomenon

Footnotes to Table IV

* Brand mean ± standard deviation, ** overall mean ± standard deviation

θ For each subject, the upper row of figures refers to the $1^{st}$ day, and the lower row to the $2^{nd}$ day of laboratory attendance (day 4 and day 11 of the experimental period, respectively)

$D$ mean nicotine input at mouth-level per cigarette

$\dot{D}$ mean nicotine input at mouth-level per hour

$c_{nic}^{24}$ mean circadian nicotine plasma concentration

$c_{cot}^{24}$ mean circadian cotinine plasma concentration

$CL_{nic}/f_L$ total nicotine clearance divided by its bioavailability

$CL_{cot}$ total cotinine plasma clearance

$EL_{cot}$ mean circadian cotinine elimination rate = $CL_{cot} \cdot c_{cot}^{24}$

$f_L*f_M$ nicotine availability * fraction converted into cotinine

$D*f_L$ mean systemic (blood-level) nicotine input per cigarette, $f_L$ is calculated from the fraction product $f_L*f_M$ for $f_M = 0.75$
TABLE IV. NICOTINE INPUT AT MOUTH- AND AT BLOOD-LEVEL, CIRCADIAN PLASMA LEVELS AND CLEARANCES OF NICOTINE AND COTININE, ELIMINATION RATE OF COTININE, AND SYSTEMIC AVAILABILITY OF NICOTINE TIMES ITS FRACTIONAL CONVERSION INTO COTININE IN THE SUBJECTS (see footnotes)

<table>
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<tr>
<th>Brand</th>
<th>Subj</th>
<th>Cnim</th>
<th>C26nic</th>
<th>C26cot</th>
<th>CLnic</th>
<th>CLcot</th>
<th>ELcot</th>
<th>fL*M</th>
<th>D*fL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/cig</td>
<td>mg/hr</td>
<td>mg/l</td>
<td>%/hr</td>
<td>mg/l</td>
<td>%/hr</td>
<td>mg/hr</td>
<td>mg/cig</td>
</tr>
<tr>
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<td>2.31</td>
<td>48.5</td>
<td>47.6</td>
<td>351</td>
<td>-</td>
<td>1.46</td>
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<td></td>
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<td>2.65</td>
<td>2.10</td>
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<td>45.6</td>
<td>338</td>
<td>4.15</td>
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<td>0.61</td>
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<td>36.1</td>
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<td>1.87</td>
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<td>16.8</td>
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<td>247</td>
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<td>1.22</td>
<td>16.7</td>
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<td>326</td>
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<td>± SD*</td>
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<td>0.60</td>
<td>10.9</td>
<td>12.4</td>
<td>81</td>
<td>0.53</td>
<td>0.26</td>
<td>0.11</td>
</tr>
<tr>
<td>V2</td>
<td></td>
<td>0.72</td>
<td>0.83</td>
<td>14.1</td>
<td>56.7</td>
<td>200</td>
<td>-</td>
<td>0.78</td>
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<td>0.95</td>
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<td>79.4</td>
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<td>0.85</td>
<td>11.0</td>
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<td>0.41</td>
<td>11.0</td>
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<td>0.39</td>
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<td>6.8</td>
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<td>-</td>
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<td>59.1</td>
<td>103</td>
<td>3.24</td>
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<td>0.71</td>
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<td>10.4</td>
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<td>3.50</td>
<td>0.49</td>
<td>0.71</td>
</tr>
<tr>
<td>± SD*</td>
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<td>0.25</td>
<td>0.25</td>
<td>2.9</td>
<td>15.8</td>
<td>58</td>
<td>0.30</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>MEAN**</td>
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<td>62.2</td>
<td>3.56</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>± SD**</td>
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<td>13.1</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
FIGURE 3 Nicotine deliveries of the two ventilated cigarette brands studied

\[ D^* = \text{nicotine yield by standard machine-smoking procedure} \]

\[ D^{**} = \text{nicotine intake at mouth-level and at blood-level, respectively, by the subject populations of the two brands} \]

Numbers given are mg of nicotine per cigarette. Mean values and standard deviations have been indicated numerically and graphically (cf fig 2, chapter 8)

Average nicotine deliveries to the subjects, both at mouth-level and at blood-level, significantly differed from the machine-derived deliveries, both for brand V1 (\( P < 0.0005 \)) and for brand V2 (\( P < 0.01 \)). Average subject nicotine deliveries of V1 and V2 as well differed from each other (\( P < 0.01 \))

may very well explain in part the high average relative nicotine retention in the filters of V1 and V2, compared to the filters of the conventional brands

The nicotine amounts retained in the filters of V1 and V2 when machinesmoked under the conditions specified (see figure 1) cover the amounts retained in the filters of cigarettes smoked by our subjects, with the excep-
tion of subject 26 Filters of the butts saved by this subject contained more nicotine than any of the machine-smoked cigarette filters. Subject 26 probably did not employ larger puff volumes than 75 ml (the highest machine puff volume employed in this study), but rather may have puffed more frequently than once a minute (the machine-smoking frequency).

In spite of the large prediction interval of D for given F (figure 1), application of the smoking machine-derived regression line to predict human intake at mouth-level appears to work out well, producing no clear-cut artifacts in total nicotine clearance and the fraction product $f_L f_M$. The values of these latter quantities compare well to those obtained in the conventional filter cigarette study. Actual computing errors probably are smaller than indicated by the prediction limits, presumably because structural positive and negative deviations from the regression lines as observed to result from rigid machine-smoking patterns are due to cancel in fluctuating intra-individual smoking technique. The existence of such fluctuations is evident from individual highly variable CO yields of single within-day cigarettes to our subjects, and at longer term from the variability of mean daily nicotine content of subject-smoked butts. For all subjects, highest and lowest mean nicotine content, as averaged over 10 butts analyzed per day, diverged by a factor 1.5-2 over the 16 days of the experimental period, as also was observed for smokers of conventional filter-tipped cigarettes in part I of this study.

Filter nicotine content, number of cigarettes smoked, the product of nicotine content and number of smoked cigarettes, and butt length did not differ between each of the two days of subjects' laboratory attendance on the one hand, and the 14 days of free movement on the other. Once more, we conclude that the experimental setting used in this study does not notably affect consumption rate and smoking technique of our subjects.

Comparing the conventional cigarette brands studied (F1-F4) to the ventilated brands studied presently with regard to mean mouth-level nicotine yield (D), it is recalled from the section 'Results' that brands V1 and F4 rank at the top of the list, whereas V2 definitely is the brand poorest in nicotine delivery (compare "D" in figure 3 of the present chapter to figure 2 in chapter 8).

It is instructive also to compare the total amount of nicotine distilled by the smokers of the 6 brands from the tobacco of their cigarettes in the act of smoking (i.e., the amount retained in the filter F, augmented by the amount passing through it and reaching the smoker's mouth D). These amounts, averaged over the smokers' population of each brand, read as follows $F + D = 2.32$.
(F1), 1 56 mg (F2), 2 29 mg (F3), and 2 45 mg (F4), 3 38 mg (VI), and 2 25 mg (V2) Here VI heads the list with distance, V2 now being indistinguishable from F1, F3, and F4.

An explanation for the high value of (F + D) for brand VI could be that the tobacco variety or blend utilized in VI production might be particularly rich in nicotine, which would be in accordance with the significant inverse correlation found by Benowitz et al between nicotine concentrations in tobacco of 15 brands and the amount of nicotine delivered in machine-smoking of these brands under standard conditions. This hypothesis is supported by the ratio of total nicotine to carbon monoxide (CO) delivery (\((F + D) / D\)) see chapter 10, figure 2), which is higher for VI than for any other of the investigated brands.

The (F + D)-value of brand V2 does not give occasion to suspect a nicotine-rich tobacco blend to be used in its manufacturing. The conspicuously low mouth-level nicotine delivery D of this brand seems entirely attributable to the high efficiency of the filters of V2 with respect to nicotine retention. This high efficiency may be due in part to the occurrence of ventilation (see the beginning of this section). Cross-sections of the proximal ends of V2-cigarettes as smoked by our volunteers indeed showed tar staining patterns characteristic of a substantial (though certainly not nominal) ventilation rate.

In literature, reductions in nicotine exposure have been reported to occur in smokers after short-term experimental switching from relatively high tar/nicotine brands to ultra low yield ventilated brands, or to cigarettes provided with ventilated cigarette holders, after spontaneous switching to ventilated-filter cigarettes in smoking cessation programs, and in habitual smokers of ventilated-filter cigarettes as compared to habitual smokers of higher nicotine yield brands. Extent of reduction varied from slight (<10%) to substantial (56%), but in all studies was less than the extent that would be expected on the basis of machine-determined nicotine yields of the brands switched from and to. Thus, like our subjects, literature subjects compensated for reduced concentrations of smoke constituents in the mainstream smoke of their cigarettes. Reduced availability of nicotine is generally viewed as the motivation to compensate, although one report states tar compensation to govern smoking behaviour over and above nicotine compensation. The array of compensatory actions through which human smokers manage to extract more nicotine from their cigarettes than the smoking machine does, may be constituted of (a combination
of one or more of) the following factors: development of a smoking technique characterized by more frequent and/or more voluminous puffs, or by deeper inhalation and/or longer breathholding (lung exposure) times, by smoking a greater length of tobacco rod, by the (unconscious or intentional) blockade of the ventilatory mechanism by smokers' lips and/or fingers, or merely by increase of cigarette consumption rate.

In a recent study, Zacny et al. pointed out that the increased number and volume of puffs taken from ventilated cigarettes may as well be due to changes in the physical characteristics of these cigarettes (resistance to draw), and not to smokers actively compensating for reduced smoke constituent yields. Whatever its main determinant, we realized that puff volumes appear to be greater for smokers of ventilated cigarettes than for those of conventional cigarettes, and added 75 ml as a puff volume in machine smoking of brands VI and V2 compared to 60 ml as the largest puff volume in machine smoking of the conventional brands F1-F4.

Our subjects smoking VI and V2 extracted considerably more nicotine from their cigarettes than the smoking machine does in the standardized procedure. This obviously demonstrates that humans may smoke in a way that is different from the machine standard. Our subjects tended to compensate for the low nicotine yield produced under standard conditions by one or more of the possibilities to do so.

The present experimental protocol, involving two days of laboratory attendance and analysis of 30 plasma samples for nicotine and cotinine (and cotinine-D2 in 15 of them), and of 16 daily portions of butts for cigarette length and nicotine content, for obvious reasons does not lend itself to be employed in large-scale surveyal studies. Adaptations to suite it for studies at large scale go without saying, and will be applied in future research.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the continuous interest and financial support provided by the W.A.R.G. (Scientific Advisory Council for Smoking and Health), and the smokers of the ventilated brands for their willing cooperation.
1 Teeuwen HWA (1988) Clinical pharmacokinetics of nicotine, caffeine, and quinine A systems dynamics approach (a) 'Nicotine intake by smokers of filter cigarettes Part I conventional filter cigarettes' (chapter 8), (b) 'Simultaneous estimation of nicotine and cotinine levels in biological fluids using high-resolution capillary-column gaschromatographic combined with solid-phase extraction work-up' (chapter 2), (c) 'Capillary gaschromatographic - mass spectrometric - selective ion monitoring method for measuring relative levels of cotinine and orally administered cotinine-D2 in smokers' blood plasmas' (chapter 4), (d) 'Estimation of the nicotine content of cigarette filters and glass fibre ('Cambridge') filters' (chapter 3), (e) 'Carbon monoxide intake by smokers of conventional and ventilated filter cigarettes' (chapter 10) Ph D Thesis, University of Nijmegen, Nijmegen, The Netherlands

2 ISO 3402 Tobacco and tobacco products Atmospheres for conditioning and testing (1972)

3 ISO 3308 Tobacco and tobacco products Routine analytical cigarette-smoking machine Definitions, standard conditions and auxiliary equipment (1977)


5 Robinson JC, Young JC, Rickert WS A comparative study of the amount of smoke absorbed from low yield ('less hazardous') cigarettes Part 1 non-invasive measures Br J Addict 1982,77 383-97


Rawbone RG, Murphy K, Tate ME, Kane SJ. The analysis of smoking parameters, inhalation and absorption of tobacco smoke in studies of human smoking behaviour. In Thornton RE (ed), Smoking behaviour - Physiological and psychological influences. Churchill Livingstone, New York (1978) 259-76


Burking TA, Stitzer ML, Bigelow GE, Mead AM. Smoking topography and carbon monoxide levels in smokers. Addict. Behav. 1985, 10: 319-23
Chapter 10

CARBON MONOXIDE INTAKE BY SMOKERS OF CONVENTIONAL AND VENTILATED FILTER CIGARETTES

A pending public health issue is whether the newly marketed ultra-low tar/nicotine/carbon monoxide yield ventilated filter cigarettes are less hazardous than others and whether smokers who cannot quit smoking should be advised to switch to these ultra-low cigarettes. We studied carbon monoxide exposure in habitual smokers of conventional and ventilated filter cigarettes in order to resolve the issue with respect to this toxic constituent of tobacco smoke.

Carbon monoxide (CO) is an odorless, colorless, asphyxiating, gaseous compound commonly resulting from incomplete combustion of organic matter. It is present in the mainstream smoke of cigarettes in a volume percentage of 1 - 4, depending on substance factors, ventilation, and smoking technique. Upon inhalation of the smoke, CO is distributed throughout the lungs and into the alveoli. Here the alveolar air CO (CO\textsubscript{alv}) is absorbed into the pulmonary blood and combines with hemoglobin to form carboxyhemoglobin (COHb). Very quickly a CO\textsubscript{alv} / COHb equilibrium is reached, which is the basis for the direct correlation found between the two forms of CO.

In two earlier studies\textsuperscript{1,2}, we investigated the nicotine yield to smokers of (4 brands of) conventional cigarettes and (2 brands of) ventilated filter cigarettes. In these studies, we also determined the CO yield to smokers, by analyzing CO in their expired alveolar air post and pre smoking single cigarettes. The CO yields of the two classes and the various brands of cigarettes will now be reported, compared with each other, and related to the nicotine yields.

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The harmful effects of CO in humans appear to be related to two characteristics of CO. First, CO has an extremely strong affinity for the oxygen transporting agent in the blood, hemoglobin. Inspired CO interferes with oxygen transport in two ways. It competes with oxygen for hemoglobin binding sites. In addition, combination of part of the hemoglobin with CO makes the rest of the hemoglobin in the capillary circulation cling to its oxygen with abnormal tenacity, causing impaired oxygen release to organs and tissues. The impaired oxygen transport ultimately produces a relative oxygen 'starvation' in the heart, coronary vessels, and other tissues.

The second destructive characteristic of CO pertains to its effect on the blood vessel walls. The CO serves to increase the permeability of the vessel wall, resulting in fluid buildup and reduced resistance to cholesterol deposits.

Indeed, elevated CO levels have been associated with increased risks to health, including increased risk of ischemic heart disease, decreased exercise tolerance, especially in those with angina pectoris, decreased cardiac contractability in persons with coronary heart disease (CHD), impairment of survival rate in patients with acute myocardial infarction, and atherosclerosis. In fact, individuals with COHb concentrations greater than 5% have a 20 times greater risk of developing atherosclerosis than individuals with concentrations less than 3%. Carbon monoxide, possibly aided by nicotine, has been found to be related to the incidence of CHD, and CHD sudden death, the most significant cause of mortality in persons who smoke. In addition, CO has been cited as one of the prime contributors to respiratory diseases such as chronic obstructive pulmonary disease (COPD), and has been associated with fetal damage.

While representing a health hazard to all of us, CO is a particularly important risk factor for smokers, whose role in the pathogenesis of the above-mentioned smoking-related diseases long has been underestimated, attention being focused on nicotine and tar. Even now that product law makes it mandatory upon cigarette manufacturers to mention the brand's standard smoking machine yield of nicotine and tar on the packet in many countries, the standard CO yield remains unmentioned in most of these countries, including The Netherlands.

We investigated and compared the CO yields of two classes of filter cigarettes: conventional and ventilated filter cigarettes.
Subjects. The subjects were 35 apparently healthy volunteers (8 males, 27 females), habitually smoking 22.1 ± 2.1 cigarettes of one brand per day. Subject's characteristics, and their distribution among the two classes and six brands of filter cigarettes have been given earlier1,2.

The characteristics of their cigarette brands have been summarized in Table I. Written informed consent to participate in the experiments was obtained from all subjects.

**TABLE I. CHARACTERISTICS OF THE CIGARETTE BRANDS SMOKED BY THE SUBJECTS**

<table>
<thead>
<tr>
<th>Brand</th>
<th>Tar mg</th>
<th>Nicotine mg</th>
<th>CO CO2</th>
<th>Filter length</th>
<th>Wrapper length</th>
<th>Cig. length</th>
<th>Rnic</th>
<th>VRnom</th>
<th>VRmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>16</td>
<td>1.2</td>
<td>17.5</td>
<td>22</td>
<td>26</td>
<td>84</td>
<td>32</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F2</td>
<td>16</td>
<td>1.1</td>
<td>16.2</td>
<td>18</td>
<td>22</td>
<td>80</td>
<td>35</td>
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<td>NA</td>
</tr>
<tr>
<td>F3</td>
<td>16</td>
<td>1.1</td>
<td>15.0</td>
<td>21</td>
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<td>84</td>
<td>34</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F4</td>
<td>13</td>
<td>1.0</td>
<td>12.0</td>
<td>21</td>
<td>25</td>
<td>84</td>
<td>32</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>V1</td>
<td>1</td>
<td>0.2</td>
<td>1.3</td>
<td>27</td>
<td>32</td>
<td>84</td>
<td>52</td>
<td>81</td>
<td>6</td>
</tr>
<tr>
<td>V2</td>
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<td>32</td>
<td>84</td>
<td>68</td>
<td>64</td>
<td>3</td>
</tr>
</tbody>
</table>

@ Stated by the manufacturer according to ISO 3308, ISO 3402, and ISO 8454.
@@ Rnic = mean relative filter retention of nicotine during machine-smoking under specified conditions (ref. 1, 2), VRnom and VRmin = nominal (maximum) and minimal ventilation rate, resp. (ref. 1, 2), NA = not applicable.

Experimental design and sampling. The experimental protocol has been described before with respect to butt collection and venipuncture schedule1. Subjects attended the laboratory at two consecutive Tuesdays from 9.00 a.m. to 17.00 p.m., during 15 minutes in the evening of these days (between 21.30 and 24.00 p.m.) and during 15 minutes (9.00 - 9.30 a.m.) in the morning of the subsequent Wednesdays. Subjects were instructed not to smoke at both Tuesdays and both Wednesdays until the first sample of breath had been produced. Otherwise they were allowed to smoke ad libitum. Twenty paired samples of expired air were obtained 1 min before and 4 min after the smoking of 10 single cigarettes, including the first-of-the-day cigarettes at the 4 days mentioned and 6 cigarettes smoked at ≈ 11.20 a.m., 14.30 p.m. and 17.00 p.m. at both Tuesdays. In the course of the latter days, unpaired samples of expired air.
not related to the smoking of any particular cigarette) also were obtained, at 10 10 a m , and 12 30 p m , 15 45 p m , and 21 30 p m

The time interval elapsing between the last puff and the production of the post-smoking breath sample was chosen 4 min as pilot experiments gave evidence of a biexponential decay of CO in alveolar air after cigarette smoking, the first (rapid) phase being extinguished 4 min after smoking at the most

Expired air samples were produced according to a breath holding technique described by Jones et al 3 Its principle involves use of the alveoli as aerotonometers until CO gas pressures of pulmonary blood and alveoli approach equilibrium After maximum inspiration from residual volume, subjects held their breath for 20 sec and then exhaled partially into a 2 l polyvinyl anaesthesia bag Exhalation was interrupted for a moment to switch bags, and an end-expiratory (alveolar) breath sample of 1-2 l was collected in a second 2 l anesthesia bag that previously had been emptied by wringing, and which was equipped with a valve which the subjects closed after complete exhalation The dead space of the valve caused an insignificant error (< 1%) Expired air was analyzed for its CO concentration a few minutes after production Expired alveolar air CO boosts as a result of cigarette smoking were calculated as the difference between corrected CO-concentrations (see eqn II) in paired breath samples obtained post and pre cigarette smoking

$$\Delta CO_{alv} = CO_{alv,post} - CO_{alv,pre}$$  (I)

expressed in parts per million (ppm)

Blood hemoglobin concentration (Hb) was taken the average of 6 measurements 50 μl aliquots of the first six blood samples taken in succession from each volunteer for the purpose of nicotine and cotinine plasma analysis was used for (Hb) measurements

Analytical methods Samples of expired alveolar air samples were drawn through two consecutive glass cylinders (150 x 25 mm ID) filled with granules of dry calcium chloride (Merck, Darmstadt, F R G ) and soda lime (Grace, Espernon, France) in order to absorb water vapour and carbon dioxide (CO2), respectively The remaining gas mixture was led through a portable CO analyzer containing a nondispersive infrared cell ('CO-monitor', Telegan Ltd , Kenley, U K ) with a linear response in the range of 0 - 2000 ppm of CO

Before operation, the combined CO monitor and connected filters were iteratively calibrated using air containing 103 ppm CO (B O C , London, U K ) and ambient air freed of CO by a hopcalite filter (The Mine Safety Appliances
Co, Pittsburgh, USA) until the monitor displayed both 0 and 103 ppm correctly CO reading values were corrected for the volume contraction resulting from water and CO\textsubscript{2} removal from expired air samples and the concomittal CO enrichment of the remaining gas sample according to

$$\text{CO}_{\text{alv,corr}} = \text{CO}_{\text{alv,read}} \left(1 - f_{\text{vol,CO2}} - f_{\text{vol,H2O}}\right)$$  \hspace{1cm} (II)

For the purpose of correction, end-expiratory air produced by the Jones' technique was assumed to contain a fractional volume $f_{\text{vol,CO2}} = 0.050$ of carbon dioxide\textsuperscript{3} and to be saturated with water vapour\textsuperscript{3} (partial saturation pressure of water vapour $37^\circ$ 62 mBar) The fractional volume of water vapour in the air samples was calculated according to

$$f_{\text{vol,H2O}} = 62/(\text{barometric pressure in mBar})$$  \hspace{1cm} (III)

and varied from 0.060 to 0.064 in the course of the measurements. Barometric pressure was measured with a Casella London 4456 barometer (Casella, London, UK) A single 10 l expired air sample containing 51 ppm of CO was analyzed 10 times with 1 hr interspacings during a working day, the observed coefficient of variation (CV) was 2%.

Blood hemoglobin concentration (Hb) was measured spectrophotometrically as azidemethemoglobin by absorption measurements at 565 and 580 nm\textsuperscript{4} using a Hemocue photometer (AB Leo, Helsingeborg, Sweden) calibrated at 128 g/l with the aid of a standard absorption cuvet Using six venous blood samples separately obtained from the same individual within 3 hrs, the CV was 3.5%.

Calculation of CO intake To determine the CO intake of our smokers, we made use of the empirical relationship between $\text{CO}_{\text{alv}}$ (ppm) and the percentage of Hb binding sites occupied by CO (%CO\textsubscript{Hb}) as determined simultaneously in expired air and blood of 162 smokers and 25 nonsmokers by Wald et al\textsuperscript{5}

$$\text{CO}_{\text{alv}} = 5.09 \%\text{COHb} + 2.34 \quad (n=187, r=0.97)$$  \hspace{1cm} (IV)

For our purpose, it is more convenient to replace the percentage of occupied Hb binding sites (%CO\textsubscript{Hb}) with a fraction of sites (fCO\textsubscript{Hb}) occupied by CO (%CO\textsubscript{Hb} = 1 $\equiv$ fCO\textsubscript{Hb} = 0.01)

$$\text{CO}_{\text{alv}} = 509 \text{fCOHb} + 2.34$$  \hspace{1cm} (V)
If \( \Delta f_{\text{CO}} \) and \( \Delta f_{\text{COHb}} \) change as a result of net CO absorption (e.g., during cigarette smoking) or disposition, the magnitude of the change of these quantities is related as:

\[
\Delta f_{\text{CO}} = \frac{\Delta f_{\text{COHb}}}{509}
\]  

(\text{VI})

\( \Delta f_{\text{COHb}} \) is the number of Moles of CO that have been absorbed (\( \Delta f_{\text{COHb}} > 0 \)) or eliminated (\( \Delta f_{\text{COHb}} < 0 \)) per mole of circulatory (monomeric) Hb. The total amount of Hb (moles) present in the blood can be computed by multiplication of the Hb concentration (\( \text{Hb} \)) (moles) by the total blood volume \( V_B \) (l). Subsequently, multiplication of \( \Delta f_{\text{COHb}} \) by this total amount of circulatory Hb results in the net change of the amount of CO (moles) in the blood:

\[
\Delta C_{\text{O}} = \Delta \text{CO}_{\text{alv}} \cdot \text{Hb} \cdot \frac{V_B}{509}
\]  

(\text{VII})

If the CO boost \( \Delta \text{CO}_{\text{alv}} \) is a result of cigarette smoking, and if the smoker's total blood volume is related to body weight (BW) as:

\[
\frac{V_B}{BW} = 0.08 \text{ l/kg}^{6,8}
\]  

(\text{VIII})

the CO yield of this cigarette (in moles) to the smoker may be calculated as:

\[
D_{\text{L, CO}} = \Delta \text{CO}_{\text{alv}} \cdot \text{Hb} \cdot 0.08 \cdot \frac{BW}{509}
\]  

(IX a)

or, if the CO yield is expressed in mg, (\( \text{Hb} \)) in g/l, and allocating all scalars.

\[
D_{\text{L, CO}} \text{ (mg)} = 2.73 \times 10^{-4} \Delta \text{CO}_{\text{alv}} \cdot \text{Hb} \cdot BW
\]  

(IX b)

Statistical evaluation. Variance analysis was performed using F-test (two populations) or Bartlett's test (multiple populations). Differences in two population averages were attributed (non-)significance on the basis of the unpaired Student's t-test, while Tukey's test (q-test) was used for the simultaneous evaluation of differences in multiple population averages. Both t-test and Tukey's test were applied two-tailed. Significance of differences in average age of smokers' populations of the six brands were investigated using the rank sum test of Kruskall & Wallis (H-test).
Figure 1 shows a representative circadian alveolar air concentration profile. The profile bears a natural resemblance to profiles of nicotine, observed in this study\(^1,2\). Trough levels of CO (and nicotine) were present in the morning before the first cigarette was smoked. Upon smoking, the CO level gradually increased throughout the day as a function of smoking rate and pattern, each cigarette producing a distinct boost in alveolar CO. A plateau level was reached by the afternoon and evening. Termination of smoking was followed by a gradual decline of expired air CO in all subjects. Nocturnal CO half-lifes of 4 - 6 hrs were in accordance with the findings of Peterson and Stewart\(^22\).

**FIGURE 1. Circadian alveolar air carbon monoxide concentration profile in a representative smoking volunteer (subj. 18). Cigarettes smoked by the volunteer are indicated at the figure bottom. Triangles denote cigarettes smoked at times unrelated to CO measurement, whereas arrows denote cigarettes for which alveolar air CO was measured just before and after smoking. Each smoked cigarette produces a distinct alveolar air CO boost.**
Table II lists individual blood hemoglobin concentrations and expired air CO boosts related to the smoking of a single cigarette as averaged over 10 cigarettes for each subject. Hemoglobin was normal in all subjects, although some female subjects (nrs. 11, 18, 25) exhibited rather large values, suggesting elevation due to the very habit of smoking. Average CO boosts varied widely between subjects, ranging from 1.7 to 12.5 ppm. Within subject CO boosts again were rather variable, indicating large fluctuations in smoking technique (in particular duration of inhalation, see 'Discussion') from one cigarette to the next. Intra- and inter-individual variability of CO boosts is proportionally

Table II | Mean Expired Alveolar Air CO Boosts and Blood Hemoglobin Concentrations of the Subjects

<table>
<thead>
<tr>
<th>Brand/ (Hb)</th>
<th>ΔCO&lt;sub&gt;alv&lt;/sub&gt; ppm</th>
<th>Brand/ (Hb)</th>
<th>ΔCO&lt;sub&gt;alv&lt;/sub&gt; ppm</th>
<th>Brand/ (Hb)</th>
<th>ΔCO&lt;sub&gt;alv&lt;/sub&gt; ppm</th>
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</thead>
<tbody>
<tr>
<td>Subj.nr. g/l</td>
<td>g/l</td>
<td>Subj.nr g/l</td>
<td>g/l</td>
<td>Subj.nr g/l</td>
<td>g/l</td>
</tr>
<tr>
<td>F1</td>
<td>1</td>
<td>161</td>
<td>1.8</td>
<td>14</td>
<td>148</td>
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<tr>
<td>2</td>
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<td>6.2</td>
<td>15</td>
<td>164</td>
<td>1.7</td>
</tr>
<tr>
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<td>4.4</td>
<td>16</td>
<td>162</td>
<td>9.8</td>
</tr>
<tr>
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<td>140</td>
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</tr>
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<td>19</td>
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</tr>
<tr>
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<td>7</td>
<td>131</td>
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<tr>
<td>F3</td>
<td>150</td>
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<td>12</td>
<td>3.8</td>
<td>7</td>
<td>3.7</td>
<td>7</td>
</tr>
</tbody>
</table>

Mean overall 149 ± 9 g/l, ΔCO<sub>alv</sub> = 7.0 ± 3.0 ppm.

Table II lists individual blood hemoglobin concentrations and expired air CO boosts related to the smoking of a single cigarette as averaged over 10 cigarettes for each subject. Hemoglobin was normal in all subjects, although some female subjects (nrs. 11, 18, 25) exhibited rather large values, suggesting elevation due to the very habit of smoking. Average CO boosts varied widely between subjects, ranging from 1.7 to 12.5 ppm. Within subject CO boosts again were rather variable, indicating large fluctuations in smoking technique (in particular duration of inhalation, see 'Discussion') from one cigarette to the next. Intra- and inter-individual variability of CO boosts is proportionally
### Table III. Carbon Monoxide Yields of Conventional and Ventilated Filter Cigarette Brands to the Subjects

<table>
<thead>
<tr>
<th>Brand/ Subj.nr.</th>
<th>D_{L,CO}</th>
<th>Brand/ Subj.nr.</th>
<th>D_{L,CO}</th>
<th>Brand/ Subj.nr.</th>
<th>D_{L,CO}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD*</td>
<td></td>
<td>Mean ± SD*</td>
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<td>Mean ± SD*</td>
</tr>
<tr>
<td></td>
<td>mg/cig.</td>
<td></td>
<td>mg/cig.</td>
<td></td>
<td>mg/cig.</td>
</tr>
<tr>
<td>FL</td>
<td></td>
<td>F3</td>
<td></td>
<td>V1</td>
<td></td>
</tr>
<tr>
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<td>5.9</td>
<td>14</td>
<td>7.8</td>
<td>26</td>
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<td>± SD**</td>
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<td>10.1</td>
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<tr>
<td>F2</td>
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<tr>
<td>± SD**</td>
<td>7.0</td>
<td></td>
<td>8.2</td>
<td></td>
<td>7.0</td>
</tr>
</tbody>
</table>

**OVERALL Mean ± SD**: $D_{L,CO} = 17.6 ± 8.7$ mg of CO per cigarette.

* intra-individual mean ± SD (n=10); ** inter-individual mean ± SD.

reflected in the ratio of mean and standard deviation of single cigarettes' CO yields to subjects, as shown in Table III.

In contrast to the fluctuations of (inter-)individual CO boosts and yields, brand CO yields, as averaged over the smokers of each brand, were relatively constant and ranged from 14 to 19 mg of CO per cigarette of the various brands, including the ventilated ones (Table III, figure 2). Subjects smoking the four conventional brands were delivered quantities of CO which compared reasonably with the machinal CO yield (figure 2).
FIGURE 2 Carbon monoxide deliveries of the cigarette brands studied. $D = \text{CO yield in the standard machine smoking procedure, } D_L = \text{CO intake (at systemic level) by the subject populations of each cigarette brand. Numbers are mg of CO per cigarette. Mean values and standard deviations have been indicated numerically and graphically.}$

$D_L$ of the conventional filter brands to their smokers did not differ significantly from the machinal yields, in contrast to those of the ventilated filter brands which did differ $P < 0.0001$ for brand V1, $P < 0.02$ for brand V2.

CO-deliveries of the 6 brands were not significantly different from each other.

In contrast, smokers of the ventilated brands V1 and V2 derived more than 13 times, respectively nearly 4 times as much CO from their cigarettes than does the smoking machine in the standard procedure. Differences between human intake and machinal yield of CO were strongly significant ($P < 0.0001$ for brand V1, $P < 0.02$ for brand V2).

Statistical analysis revealed no significant differences in mean age, weight, Hb concentration, CO boost, and CO intake between the subject populations of the six cigarette brands studied.
Analysis of CO in alveolar air. It has long been recognized that blood carboxyhemoglobin and expired air CO levels are directly correlated. Although the relationship between COHb and $CO_{alv}$ essentially is nonlinear, this non-linearity becomes manifest only at a relatively high CO-saturation of Hb. If $\%COHb \leq 20\%$, that is in the low range of hemoglobin saturation, correlation coefficients up to 0.98 were found by Wald et al., Jarvis et al., Cohen et al., and Ringold et al. Slopes of the regression equations relating $CO_{alv}$ to COHb given by these authors vary substantially, from 3.3 to 6.3 ppm/$\%COHb$ (cf. eqn IV). The intermediate value of 5.09 obtained by Wald et al. appeared to correlate excellently with the slope of the scattergram constructed by Jones et al. (who themselves unfortunately did not perform regression analysis on their data), and was used in this study.

The firm relationship between $CO_{alv}$ and COHb, and the development of relatively inexpensive, easy to operate, and fully portable instruments for assessing atmospheric CO levels, based on nondispersive infrared absorption by CO or on catalytic conversion of CO to $CO_2$ (in which case the conversion rate is proportional to the electrochemical current produced) have rendered expired air CO analysis an attractive means of non-invasively, yet in vivo, determining the CO-yield of tobacco products to human smokers. Its non-invasive character was particularly convenient in this study, since it allowed us to tune the venipuncture schedule entirely to the pseudo steady-state kinetics of nicotine and cotinine, and to preserve the full volume of the blood samples obtained for analysis of these compounds only. The breathhold technique was easy to perform for all subjects. CO in expired air samples was analyzed conveniently (digital reading) and rapidly (1½ - 2 min per sample) with our apparatus. Control of the filter materials for residual absorption capacity of water vapour and $CO_2$ was facile (visually) to perform. Refreshment of the filter materials was necessary every 5 operation days. Resetting of the monitor's zero level occurred whenever the instrument had not been in use for 1-5 hours. Recalibration of both the zero and the 103 ppm level was done when the instrument had been out of operation for one or more days.

Experimental Design. In developing the experimental design focussed on the determination of the absolute CO uptake of smokers, two approaches were conceivable. The first approach was followed for nicotine and its metabolite
cotinine\textsuperscript{1,2}, and departs from the balance of intake and elimination rates of smoke-inhaled substances during one characteristic period of a smoker's circadian living (and smoking) rhythm, this period being 24 hrs naturally. Elimination rate of CO at any moment equals its blood concentration times its clearance. If CO clearance were approximately constant and known, the total amount of CO eliminated during 24 hrs could be calculated as clearance times 24 hrs-averaged blood concentration. However, CO is cleared exclusively alveolarly and hence its clearance depends on ventilation rate and is known to vary greatly as a function of physical exercise\textsuperscript{21,22} Standardizing the ventilation rate by imposing physical inactivity upon the experimental subjects is imaginable, but still leaves us with the problem of not knowing the exact value of CO clearance. Moreover, we felt that 24 hrs of dictated inactivity would be too big an infringement of subjects' personal lives.

We therefore turned to the second approach, involving measurement of alveolar air CO boosts following smoking single cigarettes. This strategy is afflicted with the (minor) disadvantage that there is no way of measuring a subject's amount of extravascular hemoglobin (in spleen, skin, bone marrow, etc.), and intracellular myoglobin (of red muscles), which substances together bind 30-40\% of absorbed CO when the distribution of CO in the body has reached complete equilibrium\textsuperscript{43}

The proportion of CO combined with myoglobin (Mb) in man is estimated to be only about 5\% of that combined with Hb of the circulating erythrocytes, even when equilibrium has been reached\textsuperscript{41}. The small amount of CO which combines with Mb is believed to be related in part to its lesser quantity in the body and in part to its lower affinity for CO as compared with Hb\textsuperscript{42}

As to the extravascular Hb, CO diffusion to, and reaction with, the Hb of stagnant red cells in spleen, skin and bone marrow is estimated to be slow, distribution times being in the order of magnitude of an hour\textsuperscript{43}. Considering that this time is large compared to the mean input time of CO from a single cigarette (± 5 min), just after smoking termination the absorbed amount of CO in good approximation is still almost completely bound to circulatory Hb. Thus the use of eqn VII is validated.

The shortness of mean CO input time during single cigarette smoking renders measurement of CO boost insensitive to subjects' exposure to CO from environmental sources and also from endogenous physiologic processes. The intensity of exposure to CO from these sources tends to vary, if variable at all, at much longer term than cigarette smoking time.
Influence of sex on the magnitude of the CO-boosts. CO boosts for smokers of regular filter cigarettes in this study on the average were comparable in magnitude to those observed by Frederiksen and Martin, Russel et al.; they were slightly larger than those found by Henningfield et al., and Ossip-Klein et al., and smaller than those measured by Stepney, and Gori et al. for smokers of medium nicotine and tar yield cigarette brands. CO boosts for smokers of ventilated filter cigarettes in this study on the average were comparable with those observed by Zacny et al. for habitual smokers of unventilated cigarettes, who in the experiment smoked ultra low yield cigarettes whose filter vents had been blocked deliberately to an extent of either 50 or 100%.

A negative though nonsignificant correlation existed between blood Hb concentration and alveolar CO boost in our subjects ($r = -0.216$). The correlation between body weight and CO boost was both negative and significant ($r = -0.389$, $P < 0.05$), whereas the product of body weight and Hb concentration correlated even more significantly with CO boost ($r = -0.392$, $P < 0.01$).

Any physiological/anatomical textbook makes mention of a sex difference with respect to blood hemoglobin concentration, in that this concentration is higher in men than in women. We indeed observed such a difference (Men: $\{\text{Hb}\} = 158.4 \pm 6.8$ g/l, $n=8$. Women: $\{\text{Hb}\} = 146.0 \pm 8.1$ g/l, $n=27$, $P < 0.001$) (mean ± SD) in our subject group. Since mean body weight of our male smokers (BW = 75.0 ± 8.4 kg) was greater than that of our female smokers (BW = 56.9 ± 11.8 kg, $P < 0.0005$), it was inferred that our male subjects also had a greater blood volume, and hence a greater total amount of circulatory hemoglobin than our female subjects. Since there was no sex difference in CO intake, it might be expected that smoking a cigarette might give rise to a higher increment of hemoglobin CO-saturation and thus to a greater boost of alveolar CO in women than in men. This supposition was true. We observed in men a CO boost of 4.98 ± 3.18 ppm; in women boosts averaged 7.56 ± 2.80 ppm, $P < 0.05$ (mean ± SD).

The inverse relation between hemoglobin level and carboxyhemoglobin (and alveolar CO), and the resulting sex difference in $\Delta%\text{COHb}$ (and $\Delta\text{CO}_{\text{alv}}$) given equal CO intake have been observed only once before to our knowledge, by Russell et al. These authors remarked that this finding might have especial implications for anemic women smoking in pregnancy.

Because of the spreading of male and female smokers over the various cigarette brands studied, and because of the relatively small brand populations, the observed sex differences in weight, hemoglobin concentration and alveolar air CO boost were not significantly carried over into the brand populations.
Relationship between intake of nicotine and CO by the smokers

Insignificant correlations were found between subjects' mean intake of CO on the one hand, and that of nicotine (either at mouth-level or at systemic level, \( r = 0.10, n = 35 \)) on the other, both intake figures individually averaged over the 2 days of laboratory attendance. This low correlation suggests a loose relationship between the (inter)individual intake of the two compounds. Maybe a stronger correlation exists between the nicotine and the CO yield of individual cigarettes smoked by one person, but even at the intra-individual level a large extent of independence of nicotine and CO must be anticipated.

CO naturally is found in the gaseous phase of the smoke, whereas nicotine almost exclusively is associated with aerosolized particles. The mechanism of absorption of gaseous and aerosolized compounds in inhaled tobacco smoke differs fundamentally. In the next reasoning, inhalation depth is supposed to be sufficient for smoke constituents to reach the deeps airways.

Aerosols are rapidly deposited in the respiratory tract, they are trapped in the mucous layer or in the fluid film covering bronchi, bronchioli, and alveoli, respectively, the first time they come into collision with it. The mean deposition time is estimated to be in the order of a second. The extent of aerosol deposition, and, concomitantly, extent of nicotine absorption therefore is independent of lung exposure time (breathholding time) after the first second or so.

Absorption of CO from the alveoli into the pulmonary blood is governed by diffusion laws. Rate and extent of CO absorption therefore depend on the CO concentrations in the inhaled smoke and in the pulmonary blood, and on the time interval available for diffusive exchange. These three quantities are all subject to considerable fluctuation from one cigarette to the next. Duration of inhalation and breathholding is especially important, since exhalation abruptly removes the CO and thereby terminates its absorption process. Lung exposure time under natural smoking conditions endures only for a small fraction of the time that is required for completion of the CO absorption process. The latter time is equal to the time to reach CO blood-air equilibrium, and hence to the time of breathholding of the method adopted from Jones et al. 20 sec. Thus, in contrast to nicotine absorption, absorption of CO is dependent on lung exposure duration in the range of lung exposure durations normally employed by smokers.
Summarizing, physical separation of CO and nicotine in tobacco smoke and the resultant difference in dependence of absorption of either compound upon puff duration is likely to be the major factor underlying the observed poor correlation of nicotine and CO intake between individuals. The hypothesized dependence of CO intake on lung exposure time, and the independence of nicotine intake on it, have recently been experimentally verified by Zacny et al.\textsuperscript{28}. These authors also showed that inhalation depth is less critical than lung exposure time for the extent of CO absorption. Other factors held constant, 20\% of vital capacity is sufficient an inhalation depth to allow for rapid CO (and nicotine) absorption. Any surplus inhalation depth above 20\% does not give rise to a corresponding increment of rapidness and extent of absorption of both CO and nicotine\textsuperscript{28}.

Other authors also observed the ability of smokers to take in nicotine and CO in an independent way\textsuperscript{29,30}. The question raised by Ashton et al. in the title of their article: 'Should intake of carbon monoxide be used as a guide of intake of other smoke constituents?'\textsuperscript{29} should be answered 'no!' for all 'other' smoke constituents which are abundant in the aerosol phase of tobacco smoke.

Fluctuation of CO-yield within and between smokers, and between brands. Variability in CO yield between and within subjects smoking the same brand are large (Table II) and reveal proportional inter-individual and intra-individual differences in smoking technique and other factors. Apparently, smokers do not have a fixed technique, but rather smoke each cigarette in a different way, this way being affected by momentary circumstances of a social, psychological or pharmacological nature. At longer term, variability in smoking behaviour persists, as was shown by the highest/lowest ratio = 1\frac{1}{4} - 2 in mean daily butt nicotine content in the 16 day experimental period (see figure 2 in chapter 3).

Because of the relatively small CO-saturation of Hb in the morning (see figure 1), diffusion of CO to the pulmonary blood is facilitated in this phase of the circadian smoking rhythm. Indeed, the morning cigarettes tended to boost CO\textsubscript{Hb} and CO\textsubscript{alv} to a greater extent than did the other cigarettes (though just not significantly so), accounting for some of the observed variability.

In addition to technique and time of smoking, the substance smoked might exert great influence on CO intake of smokers. The potential CO delivery varies according to size and shape of the cigarette, the presence and type of the filter, and paper porosity, to moisture content, cut, packing density, and variety of the tobacco\textsuperscript{31,32,33}. In view of these multiple factors, that
hardly can be expected to be alike for the six brands studied, it is highly surprising to observe the similar level of CO yields of all six brands, including the two ventilated brands. This similarity may be explained by the observation of Herning et al. 34, that 'how a cigarette is smoked' determines intake and levels of cigarette smoke compounds, or, stated differently, that so-called 'topographical' factors (smoking technique) are more important than substance factors in the determination of CO-intake. A certain degree of coincidence cannot be excluded either in 'explaining' the observed similarity in CO yield.

In addition, the CO yield of the ventilated brands can only then be similar to that of the regular brands, if the smokers of the ventilated brands exhibit a smoking technique directed at compensation for the ventilation-induced low nicotine delivery of their brands. Compensation may be achieved by (a combination of) blocking the vent holes by fingers and/or lips, or by smoking more intensely, that is by taking larger puffs (facilitated by the low resistance to draw of ventilated cigarettes), more puffs, leaving a shorter butt, and by inhaling the smoke more deeply and longer than is usually done by smokers of regular filter-tipped or plain cigarettes.

Literature comparison of CO-deliveries of conventional and ventilated filter cigarettes. In literature, reductions of smokers' exposure to CO have been reported after short-term experimental switching from medium nicotine brands with conventional filters to ultra-low ventilated-filter cigarettes 51,52,49 or to cigarettes provided with ventilated cigarette holders 54, after long-term experimental switching to ultra-low cigarettes 55,56, and in habitual smokers of ultra-low cigarettes compared to habitual smokers of conventional filter cigarettes 57,58. The extent of these reductions in all studies was less than the extent that would be predicted comparing machine CO yields of the cigarettes involved, indicating compensatory smoking behaviour like in the present study.

Reduction of CO-intake, if observed at all, has been reported to be less than reduction of nicotine intake in smokers switching from high or medium nicotine/tar yield brands to brands ( ultra) low in nicotine and tar 45-51.

No reduction in CO exposure ( i.e., complete co-compensation of CO intake) as well has been reported after short-term experimental switching from medium nicotine brands with conventional filters to ultra-low ventilated-filter cigarettes by Robinson et al. on the basis on non-invasive 58 and invasive 48 measurements, to cigarettes provided with ventilated cigarette hold-
ers' after long-term experimental and natural switching to ultra-low cigarettes, and in habitual smokers of ultra-low cigarettes compared to habitual smokers of conventional filter cigarettes.

Precise comparison of the results of literature studies to those of the present study regarding the issue whether or not differences in CO exposure between smokers of conventional filter cigarettes high or medium in nicotine/tar, and smokers of ventilated cigarettes ultra-low in nicotine/tar are significant, is confounded by many factors.

As to the brand switching studies, it is not known if results of short-term experimental switching studies are valid at long term. There is only one report that deals with this question and suggests extrapolation of short-term results to the long term makes sense in case of switching from high- or medium yield to low-yield cigarettes. The authors of this report raise an interesting point in discriminating between experimental and 'natural' (spontaneous) switching, in that 'natural' switchers tend to compensate completely for nicotine, whereas experimental switchers compensate only partially. As an explanation, these authors argue that "the low-yield smokers in natural switching studies are self-selected, whereas in experimental studies all smokers are required to switch. It is possible that, under natural conditions, switching to lower yield cigarettes is maintained only by those who compensate fully and those who fail to do so revert to a higher yielding brand."

Comparison to either switch- and non-switch literature studies is further confounded by changes in smoking rate, variability in time passed between the last cigarette and sampling of blood or alveolar air, variability in breathing technique and breathholding time in sampling expired air, and the occurrence of subject feedback on his/her personal CO-levels.

While exercising some prudence in view of the small smokers' populations of the brands involved, data obtained in the present study suggest that smokers of ventilated filter cigarettes do not take up less CO than smokers of conventional filter cigarettes.
10 4 CONCLUSIONS

The CO intake of 35 smokers spread over 4 brands of conventional and 2 brands of ventilated filter cigarettes was studied in conditions of natural steady state smoking behavior. The CO yield of all six brands to their smokers was similar and amounted 14 - 20 mg of CO per cigarette. For the sake of reduction of CO intake, there probably is no reason to advise people who are unable (yet) to quit smoking to switch to ventilated cigarettes.

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Chapter 11

INTRAVENTOUS SINGLE DOSE PHARMACOKINETICS OF CAFFEINE. A SYSTEMS DYNAMICS APPROACH

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11.1 SUMMARY

The disposition kinetics of caffeine was investigated in 6 healthy volunteers after intravenous infusion of a 200 mg (2.8 - 3.2 mg/kg) dose. Plasma samples were collected over 30 hours and analyzed for caffeine using reversed-phase HPLC combined with UV detection. Pharmacokinetic analysis was performed by non-compartmental dynamic systems pharmacokinetics.

Intravenous caffeine was characterized by a mean (± SD) residence time of 6.5 ± 2.0 h, a total clearance of 7.1 ± 3.6 l/h, and a volume of distribution of 42 ± 8 l. Mean residence time ranged from 3.7 to 9.5 h, and total clearance from 4.9 to 14.3 l/h, indicating considerable inter-subject variability in caffeine disposition. Upper and lower extremes of caffeine disposition rate were encountered in a heavy smoker and in a female subject taking steroid oral contraceptives, respectively.

The extraction ratio of systemic caffeine molecules averaged 2 % per transit in our volunteers, with a mean body transit time of 7 min. The mean number of transits per caffeine molecule came to 56.

Key words: Caffeine, intravenous infusion, systems dynamics pharmacokinetics.
Caffeine (1,3,7 trimethylxanthine) on a molar basis probably is the second most widely consumed drug on earth, after alcohol. It is found in plants and shrubs of various families and genera indigenous to all continents. Many of these plants are cultivated nowadays. Man ingests caffeine most frequently as hot aqueous beverages constituted of these plants, like coffee, tea, mate, etc. Furthermore caffeine is manufactured into soft drinks (e.g., cola), coffee liqueurs, certain foodstuffs, pick-me-ups, and as an adjuvant in many non-prescriptive medications, mainly analgesics. Caffeine has been used successfully as a therapeutic in the treatment of apnea in neonates [1-5].

Notwithstanding its mass consumption, certain aspects of caffeine pharmacokinetics are rarely dealt with in literature, such as the kinetics of gastrointestinal absorption of caffeine, those of caffeine biotransformation into its most important primary dimethylxanthine metabolite paraxanthine, and the kinetics of the latter metabolite. Caffeine absorption is generally believed to be 'rapid' and 'complete', although until now only one study was specifically designed to investigate caffeine absorption kinetics [6]. Lelo et al. [7,8] were the first and until now the only investigators to report plasma paraxanthine kinetics after oral administration, and the fractional conversion of caffeine into paraxanthine.

We designed a sequence of studies as to determine the kinetics of caffeine and paraxanthine after intravenous and oral administration, the rate and extent of caffeine metabolism into paraxanthine, and paraxanthine kinetics. As the first part of the program, the kinetics of caffeine after intravenous infusion in six healthy young volunteers are presently reported.

11.2 MATERIALS AND METHODS

Subjects Six healthy caucasian volunteers (3 males and 3 females), participated in the experiments after their written informed consent was obtained according to institutional policy. Their mean age (± SD) and weight was 23 ± 1 years and 67 ± 4 kg, respectively. The subjects were assessed as being 'healthy' on the basis of a normal health history and standard physiologic tests. All of them were habitual consumers of coffee and/or tea. They were free of medication, except subject 6 who took oral contraceptives. All volunteers were nonsmokers and modest social drinkers of alcoholics, except subject 2 who consumed beer and cigarettes in substantial amounts. Subject characteristics are summarized in Table I.
The subjects were instructed to abstain from coffee, tea, chocolate, cacao, caffeine-containing soft drinks and medications, and alcohol for a 60 h period prior to the experiment, and during its course. The intravenous dose was administered at approximately 10:00 a.m. on the day of the kinetic trial. Subjects were allowed a light breakfast in the morning of this day. Otherwise, they had free access to allowed foods and drinks.

Intravenous administration and sample collection. The caffeine infusion solution was prepared by diluting 6 ml of injectable caffeine preparation (Caffeine and Sodium Benzoate injection fluid, 50 mg of caffeine/ml, lot nr 84124-F3404, Cooperative Pharmacists' Society 'De Onderlinge Pharmaceutische Groothandel', Utrecht, The Netherlands) with isotonic sodium chloride solution to produce 45 ml of infusion solution. A 30 ml aliquot was then administered via a heparin lock placed in a forearm vein at a constant rate of 1 ml/min using a Braun Perfusor® infusion pump (Braun AG, Melsungen, FRG). The remainder of the solution was stored for analysis.

Blood samples (5 ml) were withdrawn via a heparin lock in a vein of the contralateral arm at time zero (blank) and at approximately the following times after the start of the infusion: 5, 10, 15, 20, 25, 30 (at infusion stop), 45, and 60 min, and at approximately 1 h, 1 1/2, 2 h, 5, 7 1/2, 14, 21 and 31 h. Prior to the removal of each blood sample, 2 ml of blood was withdrawn through the heparin lock and discarded. The blood sample then was collected in a heparinized syringe (Monovette, 5 ml, Sarstedt). The last 4 samples were obtained by individual venipunctures.

After sampling, plasma was separated by centrifugation (1000 g for 10 min). Plasma samples were stored at -20°C pending analysis, which as a rule was performed within 1 month. No caffeine decomposition was detectable over 6 months in plasma samples stored as indicated.

Caffeine analysis in plasma and infusion fluids. Samples (1 ml) of plasma or of infusion fluids (after dilution by a factor 1000) of each subject were spiked with the internal standard (ISTD) 7-β-hydroxyethyltheophylline, and extracted with dichloromethane in a single step at pH = 12. The evaporation residue was reconstituted in the mobile phase consisting of water, methanol, acetonitrile, and THF (v/v/v = 85 6 6 3) and 200 μl/l of glacial acetic acid. The mobile phase was delivered at 40°C and at a rate of 1 ml/min to a 150 x 4.4 mm I.D. column home-packed with octadecylsilane (Chromosorb RP-18®, particle size 5 μm, Merck, Darmstadt, FRG). Effluent was monitored at 195 nm.
Retention times were 4.0 and 3.2 min for CAF and the ISTD. Coefficients of variation (CV) amounted to 3%, 4% and 8% at plasma caffeine concentrations of 5.0, 1.0 and 0.1 mg/l. The assay is a minor modification of a method described elsewhere [9].

Pharmacokinetic Analysis The plasma concentration vs. time data \((C_{IV}(t), t)\) during the 30 min (T) IV infusion were fitted to a sum of exponentials according to

\[
C_{IV}(t) = \sum_{i=1}^{m} \left( \frac{A_i}{T} \right) \left( 1 - e^{-t/T} \right)
\]

and after the infusion to

\[
C_{IV}(t) = \sum_{i=1}^{m} \left( \frac{A_i}{T} \right) \left( 1 - e^{-T/T} \right) \left( 1 - e^{-t-T/T} \right)
\]

Parameter estimates were obtained by use of the non-linear curve fitting program 'FARMFIT' [34]. A relative error equal to the coefficient of variation (CV) of the analytical procedure (see 'Caffeine analysis') was attributed to each data point (weight factor \(W_j = 1/(CV \cdot C_{IV}(t_j))^2\)). Given a set of parameters, the 'FARMFIT' program iteratively minimizes the weighted sums of squared deviations (WSS)

\[
WSS = \sum_{j} W_j \left( C_{IV,obs}(t_j) - C_{IV,calc}(t_j) \right)^2
\]

where \(n\) is the number of data points, and \(C_{IV,obs}(t_j)\) and \(C_{IV,calc}(t_j)\) are the intravenous plasma concentrations observed respectively calculated according to the fit parameters at the \(j^{th}\) time of sampling. 'FARMFIT' provides the minimal value of WSS finally obtained.

Data points with concentrations less than 0.075 mg/l (CV > 10%) were excluded from the fit procedure since their accuracy was considered insufficient to be compatible with correct data analysis.

The number of exponential functions was chosen on the basis of two criteria, the first being the Akaike Information Criterion (AIC) [10,11]

\[
AIC = n \cdot \ln(WSS) + 2p
\]

where \(p\) is the number of parameters to be estimated. The number of exponential functions yielding the lowest AIC value was considered the best representation of experimental data for each subject.
The second criterion to decide on the number of exponentials was a statistical comparison of the fits by the F ratio test [12,13]:

$$ F_{q-p,n-q} = \frac{(\text{WSS}_p/\text{WSS}_q - 1)(n-q)/(q-p)}{p < q} $$

where WSS_p and WSS_q are the weighted sums of squared deviations obtained by fitting equations with p and q parameters (p < q), respectively, to the number of n data points. This test indicates if WSS is reduced (i.e., if the goodness of fit has improved) significantly (P < 0.05) by the addition of one exponential to the fitting equation.

From the concentration coefficients (A_i) and the time constants (τ_i), total areas under the plasma concentration vs. time curve (AUC), under the time-concentration versus time curve (TAUC), and under the squared time-concentration vs. time curve (T^2AUC) were computed according to statistical moment theory [14]

$$ \text{AUC} = \sum_{i=1}^{m} A_i \tau_i, \quad \text{TAUC} = \sum_{i=1}^{m} A_i \tau_i^2, \quad \text{T^2AUC} = 2 \sum_{i=1}^{m} A_i \tau_i^3 $$

The total body transport function (or disposition function) ψ(t) was calculated as [15].

$$ \psi(t) = \frac{1}{\text{AUC}} \sum_{i=1}^{m} A_i e^{-t/\tau_i} $$

Mean residence time (MRT), variance of residence times (VRT), terminal half-life (τ_2), total clearance (CL), and apparent volume of distribution (V_{dss}) of caffeine were respectively computed as [15]:

$$ \text{MRT} = \frac{\text{TAUC}}{\text{AUC}}, \quad \text{VRT} = \frac{\text{T^2AUC}}{\text{AUC}} - \left(\frac{\text{TAUC}}{\text{AUC}}\right)^2, $$

$$ τ_2 = 0.693 \tau_2, \quad \text{CL} = \frac{\text{Dose}}{\text{AUC}}, \quad \text{V}_{dss} = \frac{\text{MRT} \cdot \text{CL}}{\tau_2^2} $$

where τ_2 is the time constant of the terminal declining phase of the concentration profile. Assuming cardiac output (CO) in our healthy subjects to average 360 l/h, the extraction ratio (E), the average number of recirculations (N_{rc}) and the mean transit time (MTT) can be calculated as [15]:

$$ E = \frac{\text{CL}}{\text{CO}} (*100\%), \quad N_{rc} = \frac{(1-E)}{E} = 1/E, \quad \text{MTT} = \frac{\text{MRT}}{N_{rc}} $$
11.3 RESULTS

Akaike's Information Criterion (AIC) and the F ratio test with a significance level of 0.05 yielded the same decisions regarding the number of exponentials used for fitting the data points. The data of subject 1 were fitted to a monoexponential function, those of the other subjects to a biexponential function. Subjects' fitted plasma profiles and curve fitting data are shown in figure 1 and Table I, respectively. The plasma concentration profile over the first 3 experimental hours of subject 6 is repeated in figure 2, together with her caffeine total body transport function \( \phi(t) \) both over 3 and 30 hrs. \( \phi(t) \) is proportional to the plasma concentration profile after an i.v. injection of caffeine in this subject and represents the frequency distribution of body residence times of caffeine [15].

Pharmacokinetic parameters of caffeine in our 6 volunteers have been listed in Table II. Plasma half-lives and mean body residence time (MRT) average about 4.5 and 6.5 h, respectively. Variance of residence times equals approximately MRT-square, which may be expected in case of essentially monoexponential kinetics. Caffeine is a low-clearance drug (\( CL = 7 \ l/h \)) and distributes throughout the total body water (\( V_{dss} = 42 \pm 8 \ l \pm 0.63 \pm 0.13 \ l/kg \)). During each circulatory transit through the body, 1.4 - 4.0 % of systemic caffeine molecules was withdrawn from the circulation in our 6 volunteers, mainly by hepatic biotransformation (average: \( E = 2 \pm 1 \ % \)). The mean time of a single transit through the body (MTT) amounts to about 7 min. The average number of body transits (\( N_{bc} \)) is more than 50, some of the molecules already being eliminated already during the first transit while others may complete several hundreds of recirculations.

Values of caffeine kinetic parameters in subjects 2 and 6 represent upper and lower extremes, respectively, of disposition rate.

---

**FIGURE 1.** (Top) Caffeine plasma concentration profiles during and after a 30 min intravenous infusion in 6 volunteers. For the sake of surveyability, individual profiles have been interspaced by 5 h. Profiles of consecutively numbered subjects have been presented from the left (subject 1) to the right (subject 6). (Bottom) Caffeine transport function \( \psi(t) \) for subject 6 depicted both over 3 and 30 h (solid lines), and the plasma concentration profile over the first 3 h in this subject (dashed line). Note the two ordinate scales and the dual abscissas.
caffeine 200 mg IV infusion over 30 min in 6 healthy volunteers

plasma concentration (mg/l)

frequency (l/h) ——— plasma concentration (mg/l)

transport function of caffeine subject 6

ψ(t) first 3 h

C(t) first 3 h

ψ(t)
TABLE I. SUBJECT CHARACTERISTICS, AND CURVE FITTING DATA OF CAFFEINE FOLLOWING I.V. INFUSION OF 200 mg OVER 30 MIN IN 6 HEALTHY SUBJECTS

<table>
<thead>
<tr>
<th>Subj</th>
<th>Sex</th>
<th>Age</th>
<th>BW</th>
<th>Med/Ind.</th>
<th>A_1</th>
<th>A_2</th>
<th>( \tau_1 )</th>
<th>( \tau_2 )</th>
<th>AUC</th>
<th>TAUC</th>
<th>T^2AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>m</td>
<td>23</td>
<td>68</td>
<td>A</td>
<td>5.81</td>
<td>-</td>
<td>5.70</td>
<td>33.1</td>
<td>188.4</td>
<td>2147</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>m</td>
<td>22</td>
<td>63</td>
<td>AA,T</td>
<td>2.59</td>
<td>2.83</td>
<td>49.6</td>
<td>4.18</td>
<td>13.9</td>
<td>51.2</td>
<td>416</td>
</tr>
<tr>
<td>3</td>
<td>f</td>
<td>24</td>
<td>72</td>
<td>A</td>
<td>9.39</td>
<td>4.00</td>
<td>4.61</td>
<td>7.89</td>
<td>32.3</td>
<td>249.1</td>
<td>3931</td>
</tr>
<tr>
<td>4</td>
<td>f</td>
<td>24</td>
<td>68</td>
<td>-</td>
<td>8.05</td>
<td>5.65</td>
<td>7.78</td>
<td>6.34</td>
<td>36.9</td>
<td>227.4</td>
<td>2881</td>
</tr>
<tr>
<td>5</td>
<td>f</td>
<td>22</td>
<td>60</td>
<td>SOC</td>
<td>310</td>
<td>5.19</td>
<td>0.13</td>
<td>6.28</td>
<td>33.3</td>
<td>204.6</td>
<td>2569</td>
</tr>
<tr>
<td>6</td>
<td>f</td>
<td>24</td>
<td>68</td>
<td>SOC</td>
<td>4.17</td>
<td>4.00</td>
<td>24.4</td>
<td>9.90</td>
<td>41.2</td>
<td>392.1</td>
<td>7749</td>
</tr>
</tbody>
</table>

**MEAN**
23 67 - 4.58 - 6.72 31.8 218.8 3282

**± SD**
1 4 - 1.17 - 1.97 9.4 109.8 2472

*BW = body weight, Med/Ind. = medication and drugs of indulgence: A = modest consumption of alcohol (± 10 g/day), AA = excessive consumption of alcohol (± 100 g/day), T = tobacco consumption (25 cigarettes/day), SOC = steroid oral contraceptives.*

---

TABLE II. PHARMACOKINETIC PARAMETERS OF CAFFEINE CALCULATED FROM THE PLASMA CONCENTRATION VS. TIME PROFILE DURING AND FOLLOWING INTRAVENOUS INFUSION

<table>
<thead>
<tr>
<th>Subj</th>
<th>( t_{\frac{1}{2}} )</th>
<th>MRT</th>
<th>VRT</th>
<th>CL</th>
<th>( V_{dss} )</th>
<th>( E^* )</th>
<th>MTT*</th>
<th>( N_{rc}^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.95</td>
<td>5.70</td>
<td>32.5</td>
<td>6.05</td>
<td>34.4</td>
<td>1.7</td>
<td>5.7</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>2.90</td>
<td>3.69</td>
<td>16.4</td>
<td>14.35</td>
<td>52.6</td>
<td>4.0</td>
<td>8.8</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>5.47</td>
<td>7.72</td>
<td>62.2</td>
<td>6.20</td>
<td>47.8</td>
<td>1.7</td>
<td>8.0</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>4.40</td>
<td>6.16</td>
<td>40.1</td>
<td>5.42</td>
<td>33.4</td>
<td>1.5</td>
<td>5.6</td>
<td>65</td>
</tr>
<tr>
<td>5</td>
<td>4.35</td>
<td>6.15</td>
<td>39.4</td>
<td>6.01</td>
<td>37.0</td>
<td>1.7</td>
<td>6.2</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>6.86</td>
<td>9.51</td>
<td>97.5</td>
<td>4.85</td>
<td>46.1</td>
<td>1.4</td>
<td>7.7</td>
<td>73</td>
</tr>
</tbody>
</table>

**MEAN**
4.65 6.48 48.0 7.15 41.9 2.0 7.0 56

**± SD**
1.36 1.97 28.4 3.57 8.0 1.0 1.3 17

\( t_{\frac{1}{2}} \) = plasma elimination half-life = 0.693*\( \tau_2 \).

*For calculation of \( E^* \), MTT*, and \( N_{rc}^* \), cardiac output was taken 360 l/h in our subjects.*
Occurrence of both monoexponential and biexponential decline of caffeine plasma profiles in different volunteers after a 30 min iv infusion of caffeine have been observed previously by Blanchard and Sawers [6]. A bird's-eye visual inspection of the plots in figure 1 reveals that they all are essentially monoexponential; one exponential outweighs the other with respect to contribution to the sequential statistical moments (> 95% < 5%, except for the 1st statistical moment (AUC) of subject 2).

Slight nonlinearities seem to exist in the plots of subjects 3 and 6, who are the slowest eliminators of caffeine, witness their relatively long plasma half-lives of elimination (Table II). Caffeine disposition in these subjects might be rate-limited by metabolic enzyme capacity, as was observed previously by Tang-Liu and colleagues [21]. However, because of the inaccuracy of the plasma concentration of the last data points (< 0.075 mg/l rejected for kinetic analysis) that are responsible for the major part of the apparent nonlinearity, and regarding the apparent weakness of the nonlinear phenomenon anyway, convexity was ignored and data were treated as being linear.

While the kinetic parameters of caffeine obtained in this study (Table II) are in good accordance with data for healthy and lean subjects reported earlier [6,7,16-21], three aspects require further comment.

In Table II the rapid disposition parameters of caffeine in subject 2 are conspicuous. Subject 2 was the only smoker among the subjects and consumed a daily average of 25 plain French cigarettes (Gauloises) with inhalation. As in nonsmokers about 95% of a caffeine dose is cleared via one of three demethylation pathways [8], and as demethylation is mediated by a polycyclic aromatic hydrocarbon (PAH)-inducible isozyme of microsomal cytochrome(s) P-450 [22], induction of P-450 activity in subject 2 by PAHs present in cigarette smoke may account at least partially for the rapid elimination of caffeine in this subject. Accelerated disposition of caffeine and related compounds, the dimethylxanthines, in smokers compared to nonsmokers has been observed before [9b, 23-26].

In addition, the apparent volume of distribution of caffeine was higher in subject 2 than in any of the other subjects, both on a liter and on a liter/kg basis (Table II). If not coincidental, the high distribution volume is probably connected with the drinking habit, rather than with the smoking habit of subject 2. As caffeine distributes over total body water, its appar-
ent volume of distribution may well be affected by state of hydration and hence by fluid intake. An estimated 5 % of fluid was taken in by subject 2 regularly throughout the day, enabling this subject to maintain a high level of hydration.

In this context it is interesting to note that Trang et al. [16] observed a strong positive correlation between total (i.e., almost exclusively hepatic) caffeine clearance and urine flow rate. Thus the rapid elimination clearance of caffeine in subject 2 may also in part be explained by his high fluid input and output rates.

Another factor that might elevate (and hence might contribute to inter-individual variability of) caffeine elimination rate is induction of metabolic enzymes by high exposure to dietary caffeine. Kalow [27] reported the habitual intake of caffeine not to alter the urinary metabolite pattern of caffeine in man, suggesting all enzymes involved in caffeine metabolism either be activated to an equal extent, or not at all. Mitoma et al. [28] demonstrated caffeine to induce mixed-function oxidase activity toward certain substrates in animals. The same might apply to other constituents of coffee.

In contrast to subject 2, caffeine elimination in subject 6 appeared to be retarded. At the time of the experiments, subject 6 was taking steroid oral contraceptives (SOC), and had been doing so for over 1 year. Several investigators established a relationship between the use of SOC and a decelerated pace of caffeine metabolism [29-31]. The latter phenomenon also was observed in pregnant women [32,33]. As both pregnant women and women on oral contraceptives have a greater estrogen and progesterone load than women not taking SOC and men, decrease in caffeine clearance may be related to the increased estrogen and/or progesterone load in (especially in the later stages of) pregnancy and while taking SOC. It is not known whether the estrogen or the progesterone component in SOC gives rise to deceleration of caffeine elimination. It was speculated that SOC suppresses the mixed-function oxidase system involved in the metabolism of caffeine [30]. The exact mechanism of suppression has remained obscure as yet.

The presently reported results of individual caffeine kinetics after intravenous administration will be applied to the problem of determining absolute caffeine bioavailability after administration as freshly brewed coffee and tea. These data will be reported in a forthcoming paper.
ACKNOWLEDGEMENTS

We thank the subjects for their excellent cooperation, Dr Anneke van Hecken and Inge de Lepeleire for their invaluable help in experiment performance, and Eric Elbers for his skillful analytical assistance. Furthermore, we are indebted to Douwe Egberts Royal Coffee, Tea & Tobacco Factories for the financial support provided.

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34 FARMFIT is a non-linear curve fitting FORTRAN computer program which was developed and is in current use at the Department of Pharmacology of the University of Nijmegen, Nijmegen, The Netherlands Information will be supplied on request by one of the authors (J ν R )
Chapter 12

BIOAVAILABILITY OF CAFFEINE IN COFFEE AND TEA, AND
DEPENDENCE OF CAFFEINE TOTAL CLEARANCE ON DOSE AND
URINE FLOW

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12.1 SUMMARY

Six healthy young volunteers were administered caffeine as 400 ml of
coffee or tea, or as a 30 ml intravenous infusion on separate occasions in
randomized order. Following each treatment, plasma samples were collected over
a 32 h period (or over 15 h after drinking tea) and analyzed for unchanged
caffeine.

The caffeine transport function \( \psi(t) \) was calculated for each subject
from the intravenous data as described previously [1a]. Absorption rate versus
time profiles for each subject and each oral treatment were computed by numeri­
cal deconvolution of \( \psi(t) \) and the plasma caffeine concentration vs time data
obtained after oral administration. Statistical moment analysis of the
absorption rate profiles yielded systemic availability and characteristic
absorption times.

Systemic availability of caffeine averaged 0.99 in coffee and 0.95 in
tea. Its mean and median absorption times were 26 and 20 min from coffee, and
23 and 19 min from tea, respectively. Differences of rate and extent of caf­
feine absorption kinetics from either beverage were not significant.
Individual volumes of distribution for caffeine fluctuated only slightly between treatments, whereas caffeine disposition kinetics exhibited substantial individual variability in the various treatments. As averaged over the 6 volunteers, means of caffeine body residence times increased in the order tea treatment > coffee treatment > i.v. infusion treatment, while means of total caffeine clearance increased in the reverse order. Mean body residence time and mean total clearance of caffeine in the latter (i.v.) treatment significantly differed from the corresponding quantities in the former (tea) treatment.

The mean administered caffeine dose was greatest in the coffee treatment, somewhat less in the i.v. infusion treatment, and was significantly greater in the two former treatments than in the tea treatment.

Means of urine flow rate during the first 10 h after dosage were significantly greater in the coffee and tea treatments than in the i.v. infusion treatment, presumably because of the 4% volume of the administered beverages.

It was concluded that caffeine total clearance and mean body residence time depend both on dose and on urine flow. The effect of intra-individual variability of caffeine clearance on calculation of systemic availability is discussed.

Key words: Caffeine, systemic availability, mean and median absorption times, numerical deconvolution, individual variability of clearance, dependence on dose and urine flow.

In spite of its world-wide mass consumption, little is known of the rate and extent of CAF absorption after oral administration. Ever since Axelrod and Reichenthal [2] in 1953, one hour after oral CAF administration to three volunteers, reported plasma CAF levels that 'closely approximated those following the intravenous administration', it was generally believed that CAF was absorbed rapidly and completely from the GI tract. Blanchard and Sawers recently confirmed this belief in the only study specifically designed to determine CAF bioavailability after oral administration having been reported so far [3].

However, the latter authors characterized absorption rate unsatisfactorily, by plasma peak concentrations and times of peak occurrence. Peak concentrations reflect dose and volume of distribution, rather than rate or extent of absorption, whereas peak times are a complex function of absorption, dis-
tribution and elimination rates and again provide little information on the
course of the absorption process

In addition, the aqueous CAF solution employed in the Blanchard and
Sawers study might differ from the xanthine beverages coffee and tea with
respect to rate and/or extent of CAF absorption insofar as the latter quanti­
ties are affected by factors of a physicochemical nature (e.g., fluid tempera­
ture, possible complexing agents present in coffee or tea, etcetera)

In the present study, CAF absorption rate vs time was calculated in 6
volunteers after drinking coffee and tea. A point-area numerical deconvolution
technique [4] was used, and proved to be a valid and convenient tool in the
characterization of absorption processes

In the course of the experiments, large intra-individual changes of CAF
disposition kinetics were encountered. The cause of these differences and
their influence on the outcome of bioavailability evaluations will be dis­
cussed

12.2 MATERIALS AND METHODS

Subjects Six healthy Caucasian volunteers (3 males and 3 females),
participated in the experiments after their written informed consent was
obtained according to institutional policy. Their mean age (±SD) and weight
were 23 ± 1 years and 67 ± 4 kg, respectively. Subjects were assessed as being
'healthy' on the basis of a normal health history and standard physiologic
tests. All of them were habitual consumers of coffee and/or tea. Subjects were
free of medication, except subject 6 who took oral contraceptives. Volunteers
were nonsmokers and modest social drinkers, except subject 2 who consumed
alcohol and cigarettes in substantial amounts. Subject characteristics are
summarized in Table I

Experimental design The six volunteers were administered coffee, tea,
or intravenous CAF in a randomized complete block design (each subject under­
going the three treatments in a unique sequence) with intervals of 2 weeks.
Subjects were instructed to abstain from coffee, tea, chocolat, cacao, soft
drinks and medications containing CAF, and alcohol for a 60 h period prior to
experiments, and during their course. Doses were administered at approximately
9:00 a.m. on days of kinetic trials.
Intravenous administration of CAF  The reader is referred to a previous report [1a] for the mode of intravenous CAF administration and the scheme of blood sample collection. It is remarked here that subjects were allowed a light breakfast in the morning of the intravenous dose. Subjects kept record of time and volume of each urine voiding.

Oral administration of CAF  The subjects were fasted from the previous evening until 2.5 h after ingestion of the oral dose. Again, they kept record of time and volume of urine voidings.

Coffee preparation and administration  A 45 g aliquot of coffee powder (Douwe Egberts Silver Brand, Douwe Egberts, Joure, The Netherlands) and 1 l of tap water was used to prepare 1 l of coffee by means of a Philips model HD 5339/A coffeemachine, which after its defection was replaced with a Douwe Egberts model Technivorm Type 71 machine. A 200 ml-volume of coffee was poured into a cup and allowed to cool down to 65°. At this temperature, five 10-μl samples of coffee were taken from the cup for dose analysis. No milk or sugar were added. The subject then emptied the cup in 5-10 draughts, taking a draught once or twice a minute (time of first draught t=0). Time and mass of each draught were recorded by the subject with the aid of a stopwatch and a digital balance. The temperature of the last draught typically was 54° (range 52° - 56°).

At t = 15 min, the subject consumed a second 200 ml cup of coffee following the same draught regimen. Again, five 10-μl samples were taken for dose analysis. The coffee cup and the subject's oral cavity were then rinsed with two additional 50 ml portions of water to ensure intake of the entire dose.

Blood samples (8 ml) were collected in heparinized syringes (10 ml, Monovette, Sarstedt) before t=0 (blank), at t=10 min (between the two cups of coffee), at approximately t = 20, 28, 36, 42, 50, and 60 min, and at t = 14, 14, 2, 4, 7, 14, 23 and 32 h. Blood samples were immediately centrifuged (10 min, 1000 g). Coffee samples (10 μl) were diluted to 1 ml with distilled water. Blood and diluted coffee samples were stored at -20° pending analysis, which as a rule was performed within one month. No detectable loss of CAF due to decomposition was found up to at least 6 months of storage at -20°.

Tea preparation and administration  One liter of tap water was decarbonated by 5 min boiling. Two tea-bags (Pickwick Chinese Melange, Douwe Egberts, Joure, The Netherlands) together containing 8 g of tealeaves were
submerged in the hot water immediately following boiling. After 5 min the bags were pulled up and down in the tea for a few s and were removed. Drinking temperature and draught regimen for tea were similar to those for coffee. Because tea contains relatively less CAF than coffee when both beverages are brewed as indicated, the volume of the tea samples taken from subjects' cups for dose analysis comprised a 25 µl volume, and blood sampling was pursued for no more than 15 h. Blood samples were taken as indicated before t=0 (blank), at t = 10, 18, 25, 32, 39, 46, 53 and 60 min, and at t = 1, 2, 3, 5, 7 and 15 h.

Caffeine analysis in plasma and beverages. Plasma samples (1 ml) were analyzed for unchanged CAF as described previously [1a,b]. Samples of beverages diluted to 1 ml by a factor 40 in case of tea and 100 in case of coffee were spiked with the internal standard (ISTD) and extracted with dichloromethane in a single step at pH = 7 9. The evaporation residue was reconstituted in the mobile phase consisting of water, methanol, acetonitrile, and THF (v/v/v/v = 90 4 4 2) and 200 µl/l of glacial acetic acid. The mobile phase was delivered at 40° and at a rate of 1 ml/min to a 150 x 4.4 mm I.D. column home-packed with ODS (Chromosorb RP-18, Merck, Darmstadt, FRG). Effluent was monitored at 254 nm UV. Retention times were 2.7, 3.4, 3.8, 5.1, and 9.3 min for theobromine, paraxanthine, theophylline, CAF, and antipyrine (ISTD), respectively. Coefficients of variation amounted to 3%, 4%, and 8% at plasma CAF (and paraxanthine) concentrations of 5.0, 10.0, and 0.1 mg/l. This assay is described in more detail elsewhere [1b].

Pharmacokinetic analysis, intravenous data. The plasma CAF concentration after i.v. administration of CAF was fitted to a sum of exponentials. The CAF transport function ψ(t) for each of the subjects was then calculated as

ψ(t) = \frac{1}{\text{AUC}} \sum_{i} A_i e^{-t/\tau_i}

[5], where AUC is the area under the plasma concentration vs time curve. Calculation of pharmacokinetic parameters of CAF after i.v. dosing is described in reference [1a].

Pharmacokinetic analysis, oral data. After peroral dosage of a drug solution as a series of delayed draughts, the resulting drug plasma concentration vs time profile of the drug is a convolution of the dosage function D(t), the absorption function \text{A}_\text{a}(t) and the body transport function ψ(t) according to...
The plasma data \( C_{\text{po}}(t), t \) were interpolated by a quasi-Hermite cubic spline \([6]\) by means of the IMSL routine IQHSCU \([7]\). The time increment used for interpolation was 0.001 h. Deconvolution of the cubic splined oral plasma profile and the body transport function \( \psi(t) \) according to the numerical deconvolution method described by Vaughan and Dennis \([4]\) yielded the convoluted dosage and absorption functions \( D(t) * H_a(t) \). Statistical moment analysis \([8]\) of \( D(t) * H_a(t) \) resulted in the oral dose \( D \) times the systemic bioavailability \( h_a \), and the summation of the mean dosage time \( \text{MDT} \) and the mean absorption time \( \text{MAT} \)

\[
D \cdot h_a = \int_0^u D(t) * H_a(t) \, dt, \quad \text{and}
\]

\[
\text{MDT} + \text{MAT} = \int_0^u t \cdot D(t) * H_a(t) \, dt / \int_0^u D(t) * H_a(t) \, dt
\]

where the upper integrand 'u' represents the end-time of absorption and the the cutting-off point of oral data included in bioavailability computation \((u = 1-2 \, \text{h}, \text{see figure 2, and 'Discussion')}\). The mean dosage time is calculable as the mass-weighted average time of the draughts

\[
\text{MDT} = \frac{\sum_{j=1}^n t_j \, M_j}{\sum_{j=1}^n M_j}
\]

where \( t_j \) and \( M_j \) are time and mass of the \( j^{th} \) draught. The MAT may be determined by subtraction of the MDT from \((\text{MDT} + \text{MAT})\). The median absorption time (MedAT), at which half the available dose has been absorbed, is given implicitly as

\[
\text{MDT+MedAT} / \int_0^u D(t) * H_a(t) \, dt = \frac{1}{2} \cdot h_a \cdot D
\]

Oral \( C_{\text{or}}(t), t \) data were also fitted to a biexponential equation

\[
C_{\text{or}}(t) = A_0 (e^{-t/\tau} - e^{-T/\tau}), \quad \tau > \tau_0
\]

Data points with concentrations less than 0.075 mg/l \((\text{CV} > 10\%)\) were not included in the curve fitting procedure, as their accuracy was considered insufficient to be compatible with correct data analysis. The area under the
plasma concentration vs. time curve (AUC) and under the time-concentration vs. time curve (TAUC) were calculated according to statistical moment theory [8] as:

\[ AUC = A(\tau - \tau_0), \quad \text{and:} \quad TAUC = A(\tau^2 - \tau_0^2) \]

The mean residence time (MRT) of CAF was estimated from the drug plasma concentration profile following absorption from the GI tract as: \( MRT = TAUC/AUC - \tau = \tau \) Total clearance (CL) and apparent volume of distribution (\( V_{dss} \)) of CAF were estimated as \( CL = D*\eta_a/AUC \), and \( V_{dss} = \tau*CL \), where \( \eta_a \) was taken unity.

For each subject and each treatment, cumulative voided urine volume was plotted vs. time. The volume voided at \( t = 10 \) h was determined by linear interpolation in this plot, and was divided by 10 to yield the mean urine flow rate (UFR₁₀) over the first 10 h following CAF dosing.

Statistical analysis. Mean values are reported with their standard deviations (SD). All tests were applied two-tailed. Mean caffeine doses in coffee and in tea were compared to the i.v. CAF dose by application of a version of t-test treating the latter dose as an external (deviationless) reference value. The difference of mean CAF dose administered as coffee and as tea was tested for significance by two-tailed t-test assuming dissimilar SDs and hence including calculation of an 'effective' number of degrees of freedom. Differences of mean systemic availability of CAF in coffee and tea were compared by t-test for pairwise observations.

Differences between means of triple sets of observations in the same volunteers (e.g., of MRT, \( t_{1/2} \), CL, \( V_{dss} \), UFR₁₀, and of \( \eta_a \) as calculated by 1) numerical deconvolution, 2) plain AUC ratio, and 3) AUC ratio corrected for individual changes of CAF clearance) were evaluated by univariate analysis of variance combined with Duncan’s multiple range test. \( P = 0.05 \) was taken the minimum level of significance.

\[ \text{FIGURE 1 (next page). Caffeine plasma concentration vs. time profiles following consumption of two cups of coffee (top) or tea (bottom) in 6 healthy subjects. Administered doses of caffeine are listed in Tables I and II. } \]
FIGURE 1 (cont'd) For the sake of surveyability, individual profiles have been interspaced by 5 h. Profiles of consecutively numbered subjects are presented from subject 1 (left) to subject 6 (right).
Subjective and side effects. Abstination periods prior to each experiment steadily effectuated head-ache and lethargy in subject 1. These abstination effects readily disappeared after experimental CAF administration. No adverse side effects resulting from CAF administration were experienced.

Intravenous administration of CAF. Pharmacokinetic parameters of CAF as calculated from the plasma concentration profile during and following i.v. infusion of CAF over 30 min have been reported and discussed previously [1a]. A selection of important parameters is included in Table I for convenience of comparison with the corresponding parameters obtained after peroral dosage of CAF.

Oral administration of CAF. Plasma concentration profiles in the 6 volunteers after drinking 400 ml of coffee and of tea are depicted in figures 1a and b, respectively. Profiles are typically 'nose-shaped', with conspicuously short half-lifes for subject 2 (smoker) in each case. CAF doses administered as coffee and as tea, and pharmacokinetic parameters of CAF after administration of these beverages have been tabulated in Table II (coffee) and in Table III (tea) for each subject.

The amount of CAF in the second cup of coffee/tea always equalled that in the first cup, indicating that CAF on a 15 min scale is stable in the hot aqueous environment of the freshly brewed beverages. The CAF dose administered to subjects 1, 4, and 5 as coffee brewed with the Philips machine was smaller than that administered to subjects 2, 3, and 6 with the aid of the Douwe Egberts machine, presumably because the latter machine sprinkled the hot water directly onto the coffee powder, whereas in the former machine the hot water was collected in a basin and from there, while cooling down, was gradually allowed to soak through the powder.

The i.v. CAF dose and the average dose administered as coffee both differed significantly from the average dose in tea (P < 0.001 in each case), but not from each other (P < 0.1) (Tables II and III). The mean dosage time (MDT) was shorter for tea than for coffee (P < 0.02). As MDT was under volunteer control, the difference probably reflects a better tolerance of tea than of coffee by subjects' empty stomachs.

Median and mean CAF absorption times came to 20 and 26 min for coffee and to 19 and 23 min for tea. Systemic availability amounted to 0.99 ± 0.04 for CAF from coffee and to 0.95 ± 0.06 for CAF from tea. Individual CAF
TABLE I SUBJECT CHARACTERISTICS, AND PHARMACOKINETIC PARAMETERS OF CAFFEINE FOLLOWING I V INFUSION OF 200 MG OVER 30 MIN IN 6 HEALTHY VOLUNTEERS

<table>
<thead>
<tr>
<th>Subj</th>
<th>Sex</th>
<th>Age yrs</th>
<th>BW kg</th>
<th>Med/Ind</th>
<th>TAUC mg*h^2/l</th>
<th>AUC mg*h/l</th>
<th>MRT h</th>
<th>t\textsubscript{1/2} h</th>
<th>CL l/h</th>
<th>( V_{dss} ) l</th>
<th>UFR\textsubscript{10} l/h</th>
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<tbody>
<tr>
<td>1</td>
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<td>68</td>
<td>A</td>
<td>188.4</td>
<td>33.1</td>
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<tr>
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<td>63</td>
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<td>13.9</td>
<td>3.67</td>
<td>4.18</td>
<td>2.90</td>
<td>14.35</td>
<td>52.6</td>
</tr>
<tr>
<td>3</td>
<td>f</td>
<td>24</td>
<td>72</td>
<td>A</td>
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<td>32.3</td>
<td>7.72</td>
<td>7.89</td>
<td>5.47</td>
<td>6.20</td>
<td>47.8</td>
</tr>
<tr>
<td>4</td>
<td>f</td>
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<td>68</td>
<td>-</td>
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<td>5.42</td>
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<td>-</td>
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<tr>
<td>6</td>
<td>f</td>
<td>24</td>
<td>68</td>
<td>SOC</td>
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<td>41.2</td>
<td>9.51</td>
<td>9.90</td>
<td>6.86</td>
<td>4.85</td>
<td>46.1</td>
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</table>

**MEAN** 23 67 - 218.8 31.8 6.48 6.72 4.65 7.15 41.9 105

± SD 1 4 - 109.8 9.4 1.97 1.97 1.36 3.57 3.80 33

\( BW = \) body weight, \( Med/Ind = \) medication and drugs of indulgence, \( A = \) modest consumption of alcohol (± 10 g/day), \( AA = \) excessive consumption of alcohol (± 100 g/day), \( T = \) consumption of tobacco (25 cig./day), \( SOC = \) steroid oral contraceptives.

\( t_2 \) and \( t_\frac{1}{2} \) denote the natural time constant and the half-life of CAF elimination, respectively \( t_\frac{1}{2} = 0.693t_2 \) For subject 1, whose \( \Psi(t) \) is monoexponential, MRT equals \( t_2 \).

Absorption rate vs time profiles are shown in figure 2. In many of the profiles the dual cup administration schedule can be recognized.

Mean body residence times (MRT) and CAF plasma elimination half-lifes (\( t_\frac{1}{2} \)) as averaged over the 6 volunteers decreased between treatments in the order i v infusion + coffee + tea, whereas CAF total clearance (CL) decreased in the reverse order. Differences of means were not significant between intravenous and coffee treatments, except for \( t_\frac{1}{2} \) which was shorter (\( P < 0.05 \)) in the latter treatment. Parameters again did not differ significantly between coffee and tea treatments. However, between intravenous and tea treatments, means of MRT, \( t_\frac{1}{2} \), and CL all three were substantially different (\( P < 0.01 \)) The apparent volume of distribution (\( V_{dss} \)) of CAF for each subject was approximately constant between treatments.

Individual variability of CAF clearance and body residence time were most prominent in subjects 3 and 6. These two subjects in the intravenous treatment showed the slowest CAF disposition kinetics (greatest MRTs and rather low total plasma clearances), which in the two oral treatments was notably accelerated by up to 50% (subject 3) and up to 90% (subject 6).
**TABLE II  PHARMACOKINETIC PARAMETERS OF CAFFEINE FOLLOWING ORAL ADMINISTRATION AS COFFEE TO 6 HEALTHY VOLUNTEERS**

<table>
<thead>
<tr>
<th>Subj</th>
<th>Dose</th>
<th>MDT</th>
<th>MAT</th>
<th>MedAT</th>
<th>hₐ</th>
<th>AUC</th>
<th>MRT</th>
<th>t½</th>
<th>CL</th>
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<th>UFR¹₀</th>
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<tr>
<td></td>
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<td>min</td>
<td>min</td>
<td>%</td>
<td>mg*h/l</td>
<td>h</td>
<td>h</td>
<td>t/h</td>
<td>l</td>
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<tr>
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<td>6.68</td>
<td>4.63</td>
<td>6.92</td>
<td>46.3</td>
<td>75</td>
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</table>

**MEAN** 253 10.4 25.9 20.3 99 27.1[**××**] 5.51 3.82 8.05 42.5 135

**± SD** 63 1.3 5.4 2.8 4 7.4[**××**] 0.92 0.64 2.99 8.5 53

@ Statistical analysis of means of kinetic parameters between coffee and infusion treatments. NS = not significant (P > 0.05), NA = not applicable, ND = not done In cases of significance, the type I error probability (P) has been indicated

**DISCUSSION**

Caffeine bioavailability and correction of its evaluation for changing disposition kinetics

This report confirms the findings of Blanchard and Sawers [3] and the prior general belief, that CAF is absorbed rapidly and virtually completely from the human gastrointestinal tract. No differences between coffee and tea with respect to either rate and extent of CAF absorption, putting a stop to speculations with respect to such differences based on limited or ambiguous data [19,20]. As Blanchard and Sawers, arriving at about the same results, administered a 200 ml CAF solution made up in distilled water and in one go, it is inferred that CAF absorption probably is affected neither by beverage temperature, nor by chemicals present in coffee or tea, nor by the volume administered or by the rate of its self-administration.
TABLE III. PHARMACOKINETIC PARAMETERS OF CAFFEINE FOLLOWING
ORAL ADMINISTRATION AS TEA TO 6 HEALTHY VOLUNTEERS

<table>
<thead>
<tr>
<th>Subj</th>
<th>Dose (mg)</th>
<th>MRT (min)</th>
<th>CL (l/h)</th>
<th>V_dss (l)</th>
<th>UFR_10 (ml/h)</th>
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<td>750</td>
<td>94</td>
<td>8.44</td>
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MEAN 67.4 804 22.8 19.4 45 24.3 4.75 3.29 9.15 40.6 122 ± SD 12.5 108 3.2 3.4 6 6.9 0.89 0.61 3.97 6.9 51

P<0.0001 NA NA NA NA ND P<0.01 P<0.01 P<0.01 NS P<0.05
P<0.001 P<0.02 NS NS NS ND NS NS NS NS NS

* As the declining part of the plasma CAF profiles is monoexponential, MRT of CAF equals the elimination time constant τ: MRT = τ = tₘₚ / 0.693

** Normalized to a 200 mg dose of CAF

©,@@ Statistical analysis of means of kinetic parameters © between tea and infusion treatments, and @@ between tea and coffee treatments. For the meaning of NS, NA, ND, and P, see the bottom of Table II.

In the literature [9-14] a 2½- to 7-fold inter-individual variability of CAF disposition kinetics was observed for healthy volunteers. In the present study and that of Blanchard and Sawers [3] in addition a 1½- to 2-fold within-subject variability of CAF disposition kinetics was established in some volunteering individuals.

As both the AUC ratio and the numerical deconvolution method of estimating bioavailability assume invariability of individual kinetics of drug distribution and disposition between experiments, estimations of availability will be falsely elevated or reduced if changes of disposition kinetics yet occur. Systemic availability of CAF in the present study as calculated by plain AUC ratio method is listed in the center columns of Table IV. Spurious results are obvious for subjects 3 and 6.

Such spurious results may be avoided by undertaking corrective actions. If the apparent distribution volume remains approximately constant for each volunteer, as is the case in the present study, the AUC ratio method may be
FIGURE 2 Profiles of gastrointestinal absorption rate \( D(t) + H_a(t) \) vs time of caffeine in 6 healthy volunteers following consumption of coffee (solid line) and tea (dashed line) on separate occasions.
FIGURE 2 (cont’d) For each subject, absorption rate profiles were calculated by numerical deconvolution of the total body transport function $\psi(t)$ with the first part (until $t = u$, at the arrow) of the plasma caffeine ...
FIGURE 2 (cont'd)  concentration vs time profile following coffee or tea consumption. Dual time intervals of \( D(t) \) (i.e., of consumption of two cups of coffee or tea) have been indicated in the upper left corner for each subject.
corrected for individual variance of disposition kinetics by multiplication by the ratio of the terminal plasma elimination time constants after intravenous and peroral dosage $\frac{\tau_{IV}}{\tau_{po}}$ [3,15,16].

Again under the assumption of constant distribution volume, evaluations of bioavailability using numerical deconvolution may be corrected for changing individual disposition kinetics by the very same procedure, or alternatively, by using oral data cut off at times absorption has stopped, rather than whole oral data. The latter approach was used presently and is valid only for combinations of drug and formulation having a mean absorption time that is small compared to the mean body residence time (MAT << MRT).

As the approach of estimating systemic availability by numerical deconvolution with incomplete oral data is entirely new to our knowledge, no criteria as yet have been developed with respect to the choice of the cutting-off point, i.e., the last (conc, time) data point after non-intravenous drug administration involved in the evaluation of systemic availability. Throughout this study, the cutting-off point was chosen the oral data point right-nearest to the time at which absorption rate for the last time deviated positively from zero by 5% of the maximum absorption rate. In practice, this criterion proved satisfactory and led to cutting-off times of 1-2 h. The exact times of cutting off have been indicated for each subject and oral treatment in figure 2.

As is revealed by comparison of left and right columns for coffee and tea in Table IV, if corrected for individual variability of disposition rate, the AUC ratio method and numerical deconvolution are equivalent with respect to evaluation of the extent of systemic availability of CAF in coffee and in tea. However, the numerical deconvolution approach in a unique way is able to describe the time course of absorption rate (see figure 2), whereas traditional compartmental approach with its assumption of a monoexponential time course of absorption rate obviously would fail.

Causes underlying individual variability of caffeine disposition kinetics

Factors that give rise to differences between experiments of CAF elimination rate within a given individual must be discriminated into two categories: random factors and experiment-related factors. Factors which are responsible for differences of elimination kinetics between individuals may also vary within any given individual. These randomly fluctuating factors include transient states of disease, phase of menstrual cycle, changing dietary influences, and fluctuating exposure to drugs and environmental chemicals amongst others. Because of their very randomness, these influences are
TABLE IV. SYSTEMIC AVAILABILITY OF CAFFEINE IN COFFEE AND IN TEA AS CALCULATED BY NUMERICAL DECONVOLUTION (h_a), PLAIN AUC RATIO (F), AND BY AUC RATIO CORRECTED FOR INDIVIDUAL CHANGES OF CAFFEINE ELIMINATION KINETICS (F')

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<td>F</td>
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<td>F'</td>
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</table>

MEAN 98.7 87.7 102.9 95.3 77.8 107.3
± SD 3.5 16.1 4.0 5.7 12.0 7.8

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<th>p&lt;0</th>
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<th>p&lt;0.02</th>
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<td>NS</td>
<td></td>
<td>---</td>
<td>---</td>
<td>NS</td>
</tr>
</tbody>
</table>

Statistical analysis of means of h_a, F, and F'. NS = not significant. In cases of significance, the type I error probability (P) has been indicated.

not likely to induce statistically significant differences of kinetic parameters between experiments.

Differences of the latter kind are mostly related to experimental methodology. Four experiment-related factors can be discerned in the present study design:

1) route of administration (i.v. and p.o.),
2) possible co-administration of pharmacodynamically or -kinetically relevant compounds (except CAF) present in coffee and/or tea,
3) dose of CAF administered in the various treatments, and
4) volume of water co-administered with CAF (500 ml p.o., 30 ml i.v.).

Comments on these four possible causes will now pass under separate review.

Ad 1. As CAF after peroral administration to man is completely absorbed and undergoes a negligible first-pass effect, it is unlikely that it will display kinetics different from those after intravenous administration. Indeed no such differences were observed by Blanchard and Sawers [3].

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Ad 2 Compounds other than CAF present in coffee or tea might exert influence on CAF disposition rate, e.g., by competition for metabolizing enzymes. The class of dimethylxanthine compounds may be excluded, since analysis of coffee and tea of the presently used brands revealed that the total amount of theobromine and theophylline constituted about 0.1% and 2.1% of the amount of CAF in coffee and tea, respectively, expressed on a molar base [1b]. Furthermore, competitive inhibition of CAF metabolism by other coffee or tea constituents would be expected to result in prolonged body residence time and diminished clearance of CAF after drinking these beverages as compared to i.v. infusion of pure CAF, whereas the results indicate the reverse.

Ad 3 Dose dependence of CAF disposition kinetics was observed after oral administration of 7.5 mg/kg as a powder by Tang-Liu et al. [12], but not after administration p.o. of various doses up to 10 mg/kg as a solution by Bonati et al. [17]. Assumption of dose dependence of CAF disposition kinetics may explain the relative shortness of MRT and \( t_{1/2} \), and the concomitant greatness of CL, after administration of the smallest CAF dose as tea. However, it does not explain why MRT and \( t_{1/2} \) are shorter, and CL is greater, after CAF administration as coffee compared to the infusion of CAF, while the doses involved in coffee drinking tended (\( P < 0.1 \)) to be higher than those intravenously administered. The assumption of dose dependence of CAF disposition furthermore seems to be incompatible with the apparent log-linear decline of CAF plasma concentration vs. time profiles observed throughout this study, whereas convex-descending profiles would be anticipated in case of dose dependence.

Ad 4 Oral doses of CAF in this study were administered with a 500 ml aqueous volume (including two 50 ml portions of water to rinse subjects' oral cavities). Intravenous doses were administered as 30 ml infusions.

The substantial water load co-administered with coffee and tea is the most probable cause of the significantly greater urine flow rate observed during 10 h after administration of either beverage than after i.v. infusion of CAF (cf. UFR\(_{10}\) in Tables I-III).

Trang et al. [18] in elderly men observed a highly significant positive correlation between urine flow rate and total clearance of CAF. As renal clearance of CAF contributes negligibly (<5%) to total clearance both at low and at high urinary flow rates [18], the above correlation apparently exists between metabolic CAF clearance and urine flow rate. As an explanation of their findings, the authors [18] suggested that the primary dimethylxanthine metabolites of CAF might reach sufficiently high concentration levels to com-
petitively retard CAF demethylation, and that increased renal excretion of these dimethylxanthine metabolites at higher urine flow rates could result in a reduction of the competition, thereby increasing metabolic (and total) clearance of CAF.

In the present study, diuresis induced by administration of ½ l of water with oral CAF doses, probably reinforced by the diuretic properties of CAF itself, via the mechanism described may have elevated dimethylxanthine excretion during at least 10 h following ingestion, a time interval covering the peak time of CAF and that of its main metabolite paraxanthine. By the time UFR returned to baseline, the total xanthine plasma concentration may already have been below the level at which nonlinear kinetics are clearly manifest. The absence of convex-declining concentration profiles after administration of coffee and tea is thus accounted for, as well as the relative rapidness of CAF elimination kinetics after coffee drinking compared to intravenous administration of CAF, in spite of a higher mean CAF dose in the former treatment.

In the light of the above hypothesis it was to be anticipated that metabolic elimination rate of CAF in subjects 3 and 6, being slowest in the intravenous treatment, would increase most with elevation of urine flow rate in the oral treatments, since saturation of demethylation-mediating enzymes and hence the occurrence of capacity-limited methylxanthine metabolism should be most prominent in these slow metabolizers. Importantly, in the Blanchard and Sawers study [3], the two subjects showing the greatest change of CAF elimination rate between experiments, also cleared CAF more rapidly after an oral dose administered as a 200 ml solution than after virtually the same dose as a 30 ml intravenous infusion. As in the present study, the latter authors and Bonati et al. [17] after oral doses of dissolved CAF did not observe convexity of CAF plasma profiles, whereas Tang-Liu et al. [12] did after oral doses of anhydrous pulverized CAF to their volunteers.

In summary, available evidence points toward CAF disposition kinetics, which are dose-dependent at the levels at which consumers of coffee and tea normally take in CAF, a dose-dependence that may be masked by the concomitant intake of substantial volumes of water. The contrary conclusion drawn by Bonati et al. [17] is arrived at on the basis of limited data and may be disputed on the basis of CAF plasma elimination half-lifes increasing with dose as presented by these very authors in Table I of the challenged article [17].
ACKNOWLEDGEMENTS

The authors wish to acknowledge the excellent cooperation of the subjects and the financial support provided by Douwe Egberts Royal Coffee, Tea, & Tobacco Factories

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Chapter 13

CLINICAL PHARMACOKINETICS OF PARAXANTHINE, THE MAIN CAFFEINE METABOLITE IN MAN

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13.1 ABSTRACT

Pharmacokinetics of paraxanthine, the main phase-I metabolite of caffeine in man, were investigated in healthy subjects after oral and i.v. administration of both caffeine and paraxanthine. Intravenous infusion of caffeine was performed first in all volunteers. I.v. infusion of paraxanthine, and oral administration of both caffeine and paraxanthine were then performed in randomized order. Pharmacokinetic analysis was performed using a non-compartmental dynamic systems approach.

Kinetics of caffeine and paraxanthine were rather similar in all volunteers. After i.v. administration, volume of distribution (52 ± 5 l) and total clearance (11.8 ± 2.2 l/h) of paraxanthine were slightly higher than those of caffeine (45 ± 9 l, and 8.9 ± 4.8 l/h resp.), whereas paraxanthine mean residence time (4.5 ± 1.0 h) and elimination time constant (4.6 ± 0.8 h) after i.v. administration were somewhat smaller than the corresponding caffeine values (5.7 ± 2.0 h, and 5.9 ± 1.9 h, respectively).

Like caffeine, paraxanthine was rapidly and virtually completely absorbed after ingestion as an aqueous solution: averaged over the 3 volunteers, mean absorption time was 22 ± 1 min, and bioavailability came to 0.94 ± 0.10. For caffeine these quantities amounted to 23 ± 6 min and 1.03 ± 0.13.
The fraction of caffeine converted into paraxanthine averaged 0.77 ± 0.07 after i.v. infusion and 0.84 ± 0.09 following ingestion of caffeine. The difference was attributed to competitive capacity-limited demethylation of caffeine and paraxanthine, combined with elevated urinary excretion of paraxanthine after oral administration of caffeine due to the higher load of water co-administered with oral caffeine. The water load also seemed to affect elimination half-lives of both xanthines.

After caffeine dosage, similarity of caffeine and paraxanthine half-lives effectuated typical convex paraxanthine plasma profiles which could be described excellently by gamma distributions of residence times.

Key words: Paraxanthine, caffeine, systems dynamics pharmacokinetics, peroral and intravenous dosing, individual variance of elimination rate, conversion fraction, gamma distribution of metabolite residence times.

Caffeine (CAF) in healthy adults is almost exclusively disposed of by biotransformation. Only about 0.5 - 5% of an oral or i.v. dose of CAF is excreted unchanged via the urine [2]. CAF metabolism is very complex, comprising many diverging and converging pathways, mediated by at least 4 distinct enzymes or enzyme systems, leading to a number of at least 17 metabolites known to date (figure 1). The metabolic route between CAF and theophylline in adults is reversible [3,4], the enzyme mediating the acetylation pathway is polymorphic [5], and at least some of the routes are capacity-limited at substrate concentrations lower than 10 mg/l [1b,6,7].

Four types of metabolic degradations of CAF and its 3 metabolites abundant in plasma theophylline (13X), theobromine (37X), and paraxanthine (PX, 17X) are known to occur: 1) N-demethylation at positions 1, 3 and 7 to yield di- and monomethylxanthines, and possibly hypoxanthine, and 2) oxidation at C-8 to yield dimethylated uric acids are mediated by mixed-function oxidases containing cytochrome(s) P-450 of human liver microsomes. Demethylation reactions are catalyzed mainly by the polycyclic aromatic hydrocarbon (PAH)-inducible fraction of cytochrome(s) P-450, also known as P1-450 or P-448, whereas C-8 oxidation is catalyzed predominantly by the non-PAH-inducible fraction of P-450 enzymes [8].

C-8 oxidation of monomethylxanthines is mediated by the enzyme xanthine oxidase, which is involved as well in the metabolism of endogenous purines which are structurally related to methylxanthines. Particularly 1-methyl xanthine seems to be a good substrate for xanthine oxidase [9,10].
FIGURE 1. Pathways of caffeine (137X) metabolism in adult man.

- pathway of major importance, —— pathway of minor importance,

P-448: N-demethylation pathway mediated mainly by microsomal mixed-function monooxygenases containing the PAH-inducible cytochrome P448 (P_{450}).
P-450: C-8 oxidation pathway mediated in part by cytochrome P-448, and in part by other, non-PAH-inducible forms of cytochrome P-450.

XO: C-8 oxidation pathway mediated by the soluble enzyme xanthine oxidase.

AT: N-acetylation mediated by the polymorphic enzyme N-acetyl transferase.

X = xanthine, U = uric acid. 1,3,7: positions of methyl groups. 137X = caffeine, 17X = paraxanthine, 13X = theophylline, 37X = theobromine.

AA3MU = 5-acetylamino-6-amino-3-methyl uracil
AF3MU = 5-acetylamino-6-formylamino-3-methyl uracil
AF13DMU = 5-(N-formylmethylamino)-6-amino-1,3-dimethyl uracil
AF1MU = 5-(N-formylmethylamino)-6-amino-1-methyl uracil
AF3MU = 5-(N-formylmethylamino)-6-amino-3-methyl uracil

[?] = unstable, possibly ring-opened intermediate arising after 7-demethylation of 17X, which in fast acetylators is rapidly acetylated, but in slow acetylators tends to reclose to form 1X.

This figure has been composed after consultation of ref. [2-10, 19-26, 49-59].
In addition to these metabolic routes, 3) cleavage of the 5-membered ring between C-8 and N-9 may take place to yield a non-acetylated uracil derivative (the mediating enzyme is unknown), or 4) between C-8 and N-7 to yield an acetylated uracil derivative. The latter reaction is mediated by the polymorphic enzyme N-acetyl transferase and occurs only for CAF and PX (figure 1) [5].

Ratios of renally excreted amounts of selected CAF metabolites may serve as an indicator of relative activity of the various hepatic enzymes. Thus, the ratio of urinary recoveries of 1U to 1X provides a measure of xanthine oxidase activity, whereas the ratio of \((\text{AF}3\text{M}U + \text{AA}3\text{M}U)\) to \((1U + 1X)\) is a marker of N-acetyl transferase activity, and hence of individual acetylator status (neglecting any contribution via 13X to 1X). The 17U to 17X ratio is an index of 8-oxidation activity by cytochrome(s) P-450. Finally, the ratio of urinary recoveries of \((\text{AF}3\text{M}U + 1X + 1U)\) to 17U is an index of PX demethylation activity vs. oxidation activity (figure 1).

CAF terminal half-life under conditions standardized with respect to CAF dose and urine flow reflects the activity of microsomal cytochrome P-448-mediated demethylation activity, which is reinforced by previous exposure to inducers (PAHs, cigarette smoke, rifampine) [11-15] and reduced by liver disease or intake of steroid oral contraceptives [16-18].

The multiplicity of enzymes involved in CAF degradation (which one could hope to monitor), the ubiquitous use of CAF, its cheapness, safety, and desirable kinetic features including rapid and complete gastrointestinal absorption, negligible first-pass and renal elimination, low plasma protein binding (20-25%), and distribution throughout total body water, render CAF a close-to-ideal test substance of liver function. Indeed, it has been proposed as such by many authors [19-25].

Paraxanthine (PX or 17X) is a central intermediate in CAF biotransformation. It is the major primary mono-demethylated metabolite of CAF, whose plasma concentration in most human subjects eventually exceeds that of CAF after a single dose of the latter compound. It shares with CAF all (four) types of metabolic degradation, and for that might serve itself as a test compound of liver function. As such, it would allow more rapid diagnosis than CAF, as CAF conversion to PX is by-passed. Thus, the mean conversion time (= mean body residence time of CAF, amounting to 4-8 h in healthy volunteers and longer in patients with liver disease) is saved.

Pharmacokinetic data on PX are scarce. Arnaud and Welsch recovered approximately 60% of orally administered PX as unchanged drug and methylated
xanthines and uric acids in the urine of 5 volunteers [26] Lelo and co-workers [27] studied plasma kinetics of CAF and its three primary metabolites after oral administration of each compound and under the assumption of complete systemic availability concluded that kinetics of CAF and PX were very similar in nature In a consecutive study [28] these authors demonstrated that the fractional conversion of oral CAF to PX was about 80%

In the present study PX kinetics following intravenous administration, and its rate and absolute extent of absorption from the GI tract are reported, and compared to the corresponding figures of CAF in the same volunteers

13.2 MATERIALS AND METHODS

Subjects Three male healthy volunteers participated in the experiments, after written informed consent was obtained according to institutional policy Volunteers were 22, 23, and 24 years old and their body weights were 62, 73, and 74 kg All subjects were free of medication Subjects 1 and 3 consumed alcohol at a modest level and did not smoke, while subject 2 was a steady smoker and an excessive drinker All volunteers were habitual consumers of both coffee and tea Subject characteristics are included in Table I 60 Hours before the start of an experiment and during its course, subjects refrained from consuming alcohol, coffee, tea, cola, chocolat, and analgesics containing CAF as an adjuvant Subjects had free access to cigarettes, drinks free of alcohol and CAF

Study design and protocol The intravenous infusion of CAF was part of a randomized cross-over sequence of kinetic trials designed to assess absolute bioavailability of CAF given as coffee and as tea in 6 volunteers About 6 months later, three of these volunteers participated in a 3x3 balanced incomplete block design of treatments (latin square), receiving 200 mg of CAF and PX as oral solutions, and 100 mg of PX as an i.v infusion over 25 min on separate occasions with 2 week intervals In the morning of days of i.v dosage, they were allowed a light breakfast, whereas they were fasted from the previous evening until at least 2½ h after oral dosing In each of the latin square treatments and after i.v infusion of CAF, subjects kept record of time and volume of each urine voiding
Intravenous administration and sample collection. Details of preparation and administration of the CAF infusion solution and blood sampling scheme have been reported before [1a] Curve fitting data and pharmacokinetic parameters of i.v. CAF have been repeated and averaged for the present 3 volunteers in Tables I and II for ease of comparison.

PX was purchased from Fluka (Buchs, Switzerland) and was purified from > 98% to > 99.9% by recrystallization in doubly distilled water. For preparation of the PX infusion solution, 150 mg of thus purified PX was dissolved in 75 ml of isotonic sodium chloride solution, the solution was rendered free of pyrogens and sterilized by filtration. A 50 ml volume was administered via a heparin lock placed in a forearm vein at a constant rate of 2 ml/min using a Braun Perfusor VI® infusion pump (Braun AG, Melsungen, FRG). The remaining 25 ml-volume was stored for dose control.

Blood samples (5 ml) were withdrawn via a heparin lock in a vein of the contralateral arm at time zero (blank) and at approximately the following times after the start of the infusion: 5, 10, 15, 20, 25 (at infusion stop), 30, 35, 45 and 60 min, and at t = 1, 1, 2, 0, 3, 0, 6, 3, 11, 23 and 27 h. Prior to the removal of each blood sample, 2 ml of blood was withdrawn through the heparin lock and discarded. The blood sample then was collected in a heparinized syringe (Monovette®, 5 ml, Sarstedt). The last 4 samples were obtained by individual venipunctures.

After sampling, plasma was separated by centrifugation (1000 g for 10 min). Plasma samples were stored at -20° pending analysis, which was performed within 2 weeks. No decomposition of CAF and PX was detectable over 6 months in plasma samples stored as indicated.

Oral administration and sample collection. A quantity of 250 mg of CAF (as caffeine monohydrate, Cooperative Pharmacists' Society 'De Onderlinge Pharmaceutische Groothandel', Utrecht, The Netherlands) or of PX (Fluka, Buchs, Switzerland) was dissolved in 125 ml of mineral water of ambient temperature. Volunteers were administered 100 ml at one go (t = 0), the remaining 25 ml volume was stored for dose control. The drinking glass and the subject's oral cavity were consecutively rinsed with 2 additional 100 ml-portions of distilled water to ensure intake of the entire dose. Blood samples were collected by repeated venipunctures before t = 0 (blank), and at t = 10, 20, 30, 40, 50, 60, and 70 min, and at t = 1, 2, 2, 3, 5, 8, 12, 24, and 32 h.
Caffeine and paraxanthine analysis in plasma and injection fluids. Plasma samples (1 ml) and samples of administered fluids diluted a factor 1000 to 1 ml were spiked with the internal standard (ISTD) β-hydroxyethyl theophylline and extracted with dichloromethane in a single step at pH = 7.9. The evaporation residue was reconstituted in the mobile phase consisting of water, methanol, acetonitrile, and THF (v/v/v/v = 90 4 4 2) and 200 μl/l of glacial acetic acid. The mobile phase was delivered at 40° and a rate of 1 ml/min to a 150 x 4.4 mm ID column home-packed with octadecylsilane (Chromosorb RP-18®, particle size 5 μm, Merck, Darmstadt, FRG). Effluent was monitored at 254 nm UV. Retention times were 3.4, 3.8 and 4.5 min for PX, the ISTD, and CAF, respectively. Coefficients of variation (CV) were similar for CAF and PX and amounted to 3%, 4% and 8% at plasma concentrations of 5.0, 1.0 and 0.1 mg/l. The assay is a minor modification of a method described elsewhere [1c].

Pharmacokinetic Analysis. For each subject and each experiment, cumulative voided urine volume was plotted versus time. The volume voided at t = 10 h was determined by linear interpolation in this plot, and was divided by 10 to yield the average urine flow rate (UFR₁₀) over the first 10 h following dosage.

Intravenous data. The plasma concentration vs time data (C₁ᵥ(t), t) of CAF and PX during the time (T) of iv infusion of the respective compounds were fitted to a sum of exponentials according to:

\[ C₁ᵥ(t) = \sum \frac{mA_1}{T} \left(1 - e^{t/T_1}\right) \]

and following the infusion to:

\[ C₁ᵥ(t) = \sum \frac{mA_1}{T} \left(1 - e^{(t-T)/T_1}\right) \]

Parameter estimates were obtained by use of the non-linear curve fitting program 'FARMFIT' [29]. Each data point was attributed a weight factor:

\[ W_j = 1/(CV \cdot C₁ᵥ(t_j))^2 \]

where CV is the coefficient of variation (CV) of the analytical procedure (see above). Given a particular fit equation, 'FARMFIT' iteratively minimized the weighted sums of squared deviations (WSS)

\[ WSS = \sum \frac{W_j \cdot \Delta C₁ᵥ(t_j)}{2} \]
where \( n \) is the number of data points, and \( \Delta C_{1V}^2(t_j) \) is the squared deviation of the measured concentration from the concentration calculated according to the fit equation at the \( j \)th time of sampling. Data points with concentrations less than 0.075 mg/l (CV > 10%) were excluded from the fit procedure since their accuracy was considered insufficient to be compatible with correct data analysis.

The number of exponential functions was chosen on the basis of two criteria, the first being the Akaike Information Criterion (AIC) [30,31]

\[
\text{AIC} = n \cdot \ln(\text{WSS}) + 2p
\]

where \( p \) is the number of parameters to be estimated. The number of exponential functions yielding the lowest AIC value was considered the best representation of experimental data for each subject.

The second criterion to decide on the number of exponentials was a statistical comparison of the fits by the F ratio test [32,33]

\[
F_{q-p,n-q} = \frac{\text{WSS}_p / \text{WSS}_q - 1}{(n-q)/(q-p)}, \quad p < q
\]

where \( \text{WSS}_p \) and \( \text{WSS}_q \) are the weighted sums of squared deviations obtained by fitting equations with \( p \) and \( q \) parameters (\( p < q \)), respectively, to the number of \( n \) data points. This test indicates if \( \text{WSS} \) is reduced (i.e., if the goodness of fit has improved) significantly (\( P < 0.05 \)) by the addition of one exponential to the fitting equation.

From the concentration coefficients \( (A_i) \) and the time constants \( (\tau_i) \), areas under the plasma concentration vs time curve (AUC), the time*conc vs time curve (TAUC) and the time²*conc vs time curve (T²AUC) were computed to infinite time according to statistical moment theory [34]

\[
\text{AUC} = m \sum_i A_i \tau_i, \quad \text{TAUC} = m \sum_i A_i \tau_i^2, \quad \text{and} \quad \text{T²AUC} = 2 m \sum_i A_i \tau_i^3
\]

The total body transport function (or disposition function) \( \psi(t) \) was calculated as

\[
\psi(t) = 1/\text{AUC} \sum_i A_i \cdot e^{-t/\tau_i}
\]

Mean residence time (MRT), terminal half-life (\( t_{1/2} \)), total clearance (CL), and apparent volume of distribution (\( V_{dss} \)) were respectively computed as
where $\tau_2$ is the time constant of the terminal declining phase of the concentration profile. Assuming cardiac output $CO$ in our healthy subjects to average 360 l/h, the extraction ratio ($E$), the average number of recirculations ($N_{rc}$) and the mean transit time (MTT) can be calculated as \[E = \frac{CL}{CO}, \quad N_{rc} = \frac{(1-E)/E}{1/E}, \quad \text{and} \quad \text{MTT} = \frac{\text{MRT}}{N_{rc}}\]

**Oral data** After rapid swallowing of the CAF and PX solutions, the plasma concentration vs time profile of either drug is a convolution of the absorption function $H_a(t)$ and the total body transport function $\psi(t)$ according to

$$C_{or}(t) = \frac{D}{CL} \ast H_a(t) \ast \psi(t)$$

where $D$ is the oral dose, $CL$ is the total clearance, and ‘$\ast$’ denotes the convolution operator. Plasma data ($C_{po}(t)$, $t$) after peroral administration of CAF and PX were interpolated by a quasi-Hermite cubic spline [37] by means of the IMSL routine IQHSCU [38]. The time increment for interpolation was 0.001 h. The absorption function $H_a(t)$ was obtained for each subject and both ingested xanthines by deconvolution of his splined oral data and the appropriate body transport function $\psi(t)$ according to the numerical deconvolution method described by Vaughan and Dennis [39]. Statistical moment analysis [34] of $H_a(t)$ yielded systemic availability ($h_a$) and mean absorption time (MAT)

$$h_a = \int_0^u H_a(t) \, dt, \quad \text{and} \quad \text{MAT} = \int_0^u t \ast H_a(t) \, dt / \int_0^u H_a(t) \, dt$$

where the upper integrand ‘$u$’ denotes the time of the oral data point right-nearest to the to the time at which the absorption rate for the last time deviated positively from zero by 5% of the maximal absorption rate ($u = 1.5$ h, see figure 3). After determination of $h_a$, the median absorption time (MedAT), at which half the available dose is absorbed, could be calculated numerically according to the implicit expression

$$\text{MedAT} = \int_0^{h_a} H_a(t) \, dt = \frac{1}{2} h_a$$

Oral ($C_{or}(t)$, $t$) data were also fitted directly to a biexponential equation.
The area under the plasma concentration vs time curve (AUC) and under the time*concentration vs time curve (TAUC) were calculated as [34]

\[ \text{AUC} = A \left( t - \tau_0 \right), \quad \text{and} \quad \text{TAUC} = A \left( \tau^2 - \tau_0^2 \right) \]

Mean residence times (MRT) of CAF and PX were estimated from the plasma concentration profiles following oral dosage as the elimination time constant \( \tau \)

**Metabolic formation of paraxanthine from caffeine** PX plasma concentration vs time profiles after either oral or iv administration of CAF were fitted to a power function of time

\[ C_p(t) = A \left( \frac{t}{\tau} \right) e^{-t/\tau} \]

where \( \kappa > 0 \) to impose the origin as a left boundary upon the function. The area under the plasma concentration vs time curve (AUC) and under the time*conc vs time curve (TAUC) were calculated as [34, 40]

\[ \text{AUC} = A \tau \Gamma(\kappa+1), \quad \text{and} \quad \text{TAUC} = A \tau^2 \Gamma(\kappa+2) \]

where \( \Gamma(x+1) = \int_0^\infty t^x e^{-t} dt, \quad x > -1 \)

denotes the mathematical gamma function (see e.g., Churchill [41]). The quotient TAUC/AUC as usual yields the sum of the mean times of all processes contributing to dispersion of the respective concentration profile [36]. In the present case, TAUC/AUC is mainly made up of three mean times, with negligible contribution from some others. These three mean times are, in reverse order the mean residence time of the metabolite PX (MRT), that of its parent compound CAF (MRT\(_p\)), and the mean input time (MIT) of CAF. The MIT equals the mean absorption time (MAT) in case of oral CAF dosage at one go (in case of delayed oral dosage MIT = MAT + MDT (mean dosage time), see ref 1b). In case of intravenous dosage, MIT equals the mean infusion time (IT) Consequently, the mean residence time of PX may be computed as

\[ \text{MRT} = \frac{\text{TAUC}}{\text{AUC}} - \text{MRT}_p - \text{MIT} \]

The conversion fraction \( f_p \) of CAF into PX was determined as
where CL\textsubscript{iv} is the total PX clearance observed after iv administration of PX to each subject, D is the oral or intravenous dose of CAF administered to each subject (200 mg, assumed to be completely available after oral dosage), and 0.928 is a factor correcting for the different molecular weights of CAF (MW = 194) and PX (MW = 180). The ratio of the PX mean body residence times after infusion of this compound as such (MRT\textsubscript{iv}) and after dosing of CAF (MRT) is employed to correct $f_M$ for individual changes of PX elimination rate between the two experiments mentioned.

**FIGURE 2** Plasma concentration vs time curves of paraxanthine in a healthy volunteer (o) after instantaneously swallowing an aqueous solution of the drug, and (*) during and following a 25 min intravenous infusion.
Intravenous administration of paraxanthine. Like in case of fitting CAF data [1a], Akaike's Information Criterion and the F ratio test (applying a minimal level of significance of $P < 0.05$) again yielded the same decisions regarding the number of exponentials used for fitting the PX data points. Data points of subjects 1 and 3 were fitted to a monoexponential equation (e.g., see figure 2, lower curve), whereas those of subject 2 were fitted to a biexponential function. Subjects' curve fitting data and pharmacokinetic parameters of PX have been tabulated in Tables I and II, respectively, in which the corresponding quantities of CAF in these subjects [1a] have been repeated for ease of comparison.

Plasma elimination half-lives and mean body residence times (MRT) of PX averaged about 3 and 4.5 h, respectively. The variance of residence times (VRT) well approximates MRT$^2$ in subject 2, emphasizing the basically monoexponential
TABLE II. PHARMACOKINETIC PARAMETERS OF CAFFEINE AND PARAXANTHINE AS CALCULATED FROM THE PLASMA CONCENTRATION PROFILES FOLLOWING I.V. INFUSION

<table>
<thead>
<tr>
<th>Subj</th>
<th>$t_f$ h</th>
<th>MRT h</th>
<th>VRT h²</th>
<th>CL l/h</th>
<th>$V_{dss}$ l</th>
<th>$E^*$ %</th>
<th>MTT* min</th>
<th>$N_{re}$</th>
<th>UFR¹₀ ml/h</th>
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<td>PX</td>
<td>1</td>
<td>3.70</td>
<td>5.33</td>
<td>28.4</td>
<td>10.9</td>
<td>58.0</td>
<td>3.0</td>
<td>9.7</td>
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<td>8.7</td>
<td>30</td>
</tr>
<tr>
<td>± SD</td>
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<td>0.95</td>
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<td>2.2</td>
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<td>0.6</td>
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<td>1.3</td>
<td>1.6</td>
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</tr>
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</table>

* For calculation of $E$, MTT, and $N_{re}$, cardiac output C.O. was taken 360 l/h

$t_f = \text{plasma elimination half-life} = 0.693 \tau^2$

Kinetics of PX in this subject in terms of statistical moments. PX distributes throughout total body water and is cleared from plasma at a rate of nearly 12 l/h. During each systemic transit of PX, on the average 3 - 4% of circulating PX molecules were extracted from our volunteers' system, either by biotransformation or by excretion. The mean time to complete one circulatory transit through the body (MTT) came to nearly 9 min. The mean number of transits per PX molecule amounted to 30, some PX molecules managing to complete only a few transits, others recirculating up to more than hundred times.

Kinetic parameters of CAF and PX were rather similar. Means of total clearance and apparent volume of distribution were some 30% and 15% higher for PX than for CAF, while MRT and $t_f$ for PX were 25 - 30% lower for PX than for CAF (Table II). Student's t-test did not reveal significant differences between CAF and PX with respect to any of the listed parameters.

Oral administration of caffeine and paraxanthine. The plasma level of PX as a function of time in subject 3 after oral administration of an aqueous solution of PX is displayed in figure 2 (upper curve); similar profiles of CAF after dosage of aqueous CAF solutions have been displayed in figure 4 for all volunteers.
FIGURE 3 Profiles of gastrointestinal absorption rate ($D \cdot H_8(t)$) vs time of paraxanthine (dashed line) and of caffeine (solid line) in 3 healthy
Absorption and disposition parameters of perorally administered PX and CAI are given in Table III. Both compounds are rapidly and virtually completely absorbed, median absorption times coming to 18-20 min, mean times to 22-23 min, and absolute availability to 94 ± 10% for PX and to 103 ± 13% for CAI as averaged over our volunteers. Absorption rate profiles of PX and CAI in each volunteer have been depicted in figure 3. Profiles of PX tend to be smoother than those of CAI.

Plasma half-lives of PX elimination were similar after oral and iv administration for subjects 1 and 3 (see figure 2), but not for subject 2, who disposed of PX considerably faster after ingestion than after iv infusion. All subjects disposed of oral CAI more rapidly than of iv CAI (also compare
### TABLE III

**ABSORPTION PARAMETERS AND MEAN RESIDENCE TIMES OF CAFFEINE AND PARAXANTHINE, AND SUBJECTS' URINE FLOW RATES FOLLOWING ORAL ADMINISTRATION OF SOLUTIONS CONTAINING 200 MG OF EITHER COMPOUND**

<table>
<thead>
<tr>
<th>PARAXANTHINE</th>
<th>CAFFEINE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subj</strong></td>
<td><em>h_a</em></td>
</tr>
<tr>
<td>1</td>
<td>105 1</td>
</tr>
<tr>
<td>2</td>
<td>89 9</td>
</tr>
<tr>
<td>3</td>
<td>86 3</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>93 8</td>
</tr>
<tr>
<td><strong>± SD</strong></td>
<td>10 0</td>
</tr>
</tbody>
</table>

* MRT here equals the single elimination time constant τ

### TABLE IV

**CURVE FITTING DATA AND PHARMACOKINETIC PARAMETERS OF PARAXANTHINE AS CALCULATED FROM THE PLASMA CONCENTRATION PROFILES DURING AND FOLLOWING INTRAVENOUS (I V) AND PERORAL (P O) ADMINISTRATION OF 200 MG OF CAFFEINE**

<table>
<thead>
<tr>
<th><strong>Subj</strong></th>
<th><strong>A</strong></th>
<th><strong>τ</strong></th>
<th><strong>TAUC</strong></th>
<th><strong>AUC</strong></th>
<th><strong>MRT</strong></th>
<th><strong>f&lt;sub&gt;M&lt;/sub&gt;</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I V</strong></td>
<td>1 1</td>
<td>2 57</td>
<td>1 16</td>
<td>5 56</td>
<td>150 0</td>
<td>15 4</td>
</tr>
<tr>
<td>2 2</td>
<td>3 04</td>
<td>0 95</td>
<td>4 02</td>
<td>93 4</td>
<td>11 9</td>
<td>3 91</td>
</tr>
<tr>
<td>3 3</td>
<td>2 07</td>
<td>1 00</td>
<td>6 59</td>
<td>179 7</td>
<td>13 6</td>
<td>5 22</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>2 56</td>
<td>1 04</td>
<td>5 39</td>
<td>152 8</td>
<td>13 7</td>
<td>5 07</td>
</tr>
<tr>
<td><strong>± SD</strong></td>
<td>0 49</td>
<td>0 11</td>
<td>1 29</td>
<td>51 5</td>
<td>1 7</td>
<td>1 09</td>
</tr>
<tr>
<td><strong>P O</strong></td>
<td>1 4</td>
<td>2 66</td>
<td>1 07</td>
<td>4 98</td>
<td>140 4</td>
<td>13 7</td>
</tr>
<tr>
<td>2 2</td>
<td>3 11</td>
<td>1 13</td>
<td>2 95</td>
<td>61 2</td>
<td>9 7</td>
<td>2 85</td>
</tr>
<tr>
<td>3 3</td>
<td>2 49</td>
<td>1 05</td>
<td>6 02</td>
<td>188 6</td>
<td>15 3</td>
<td>5 51</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>2 75</td>
<td>1 08</td>
<td>4 65</td>
<td>130 1</td>
<td>12 9</td>
<td>4 45</td>
</tr>
<tr>
<td><strong>± SD</strong></td>
<td>0 32</td>
<td>0 04</td>
<td>1 56</td>
<td>64 3</td>
<td>2 9</td>
<td>1 41</td>
</tr>
</tbody>
</table>

* τ here is an apparent elimination time constant, resulting from simultaneous PX elimination and its continued formation from CAF
plasma concentration (mg/l)

caffeine 200 mg
IV infusion over 30 min
subject 1

time (h)

plasma concentration (mg/l)

caffeine 200 mg
IV infusion over 30 min
subject 2

time (h)

plasma concentration (mg/l)

caffeine 200 mg
IV infusion over 30 min
subject 3

time (h)
plasma concentration (mg/l)

caffeine 200 mg
administered as a 100 ml solution
subject 1

plasma concentration (mg/l)

caffeine 200 mg
administered as a 100 ml solution
subject 2

plasma concentration (mg/l)

caffeine 200 mg
administered as a 100 ml solution
subject 3
the mean residence times of PX and of CAF as administered via both routes, Tables II and III)

Metabolic formation of paraxanthine from caffeine Plasma concentration profiles of CAF after oral and i.v. administration, and of PX originating from CAF through metabolism, are shown in figure 4. The metabolite curves assume a typical convex time course and all intersect the curves of their parent compound, be it at widely varying times. The convex PX profiles could not be fitted properly to sums of up to 4 exponentials, but were described excellently by the mere 3 parameters of a gamma distribution of residence times ('gamma function')

Gamma function curve fitting data and kinetic parameters after administration of the metabolic parent CAF have been listed in Table IV. Values of the time power 'κ' were close to unity in all instances. The time constant 'τ' in all but one case assumed an intermediate value between the elimination time constants of CAF and of PX administered via the route in question. Estimated molar fractions of 0.77 and 0.84 of intravenous and peroral CAF doses, respectively, were converted into PX

13.4 DISCUSSION

This is the first study describing kinetics of PX after intravenous administration to man. Kinetics of PX and CAF after i.v. infusion to the same 3 volunteers in the present study were found to be similar in nature, a similarity that was also recognized by Lelo and colleagues [27] after oral dosage to the double number of volunteers. The similarity is the result of similarities of chemical structure and physicochemical properties (affecting binding to proteins and tissue) and of metabolic degradation via the same metabolic routes by the same enzyme(s) of cytochrome P-450 (figure 1), for which both

FIGURE 4 (previous two pages) Plasma concentration vs time profiles of (Δ) caffeine and of (ο) paraxanthine (p 245) during and following i.v. infusion of caffeine over 30 min, and (p 246) after swallowing an aqueous solution containing the same dose of caffeine. Caffeine profiles have been fitted to a biexponential function, those of paraxanthine to a single 'gamma' function
xanthines probably have alike affinities.

After ingestion as a solution, absorption kinetics of PX also resembled that of CAF. Rate and extent of CAF absorption data reported here are in agreement with more extensive data derived in a previous study on this subject [1b]. Mean systemic availability of PX after oral dosage was lower than that of CAF, but did not differ significantly from it or from unity. Our findings probably support the validity of the assumption of complete availability of oral PX as it was made by Lelo et al. [27], though it must be bore in mind that the latter authors administered a gelatin capsule with PX rather than a solution. Although not discernable from figures on overall rate and extent of absorption, profiles of absorption rate vs. time for PX apparently were smoother than for CAF. The significance of this finding is unclear.

PX plasma concentration vs. time functions after intake of CAF could well be fitted to a single gamma function. The gamma power function of time was originally derived by Wise [42-44] to assess the kinetics of bone-seeking elements, and which function was interpreted in terms of combined diffusive and convective motion of administered molecules ('random walks with drift'). An excellent paper on the application of gamma distributed residence times in pharmacokinetics has been published by Weiss [40].

For the first time to our knowledge, in the present study gamma functions have been applied to metabolite kinetics. Van Rossum et al. have shown theoretically that the concentration of a primary metabolite (e.g., PX) may be seen as the output of a metabolic system in which the parent compound (e.g., CAF) concentration is the input [36]. Since the transport functions $\psi(t)$ of both PX and CAF are largely monoexponential and have similar time constants, the PX concentration following a short (pulse-like) input of the parent compound CAF should follow the profile of a gamma function with $\kappa = 1$.

In the experimental practice of the presently described work, slight deviations of $\kappa$ from unity are observed. These may be explained on the basis of slight differences between the elimination time constants $\tau$ of CAF and PX. Also, the mean input time of CAF by i.v. infusion or gastro-intestinal absorption may not be short (pulse-like) enough to be negligible compared to the mean residence times of CAF and PX, elevating dispersion of the PX profile, resulting in a value of $\kappa > 1$.

It is stressed here that the gamma function as a description of PX kinetics after CAF dosage as presented above was arrived at under the assumption of linear pharmacokinetics. Thus, the convexity of the plasma PX concentration profile is solely due to similarity of the elimination time constants of CAF and PX. The log-nonlinear course obviously should not in itself be mis-
interpreted as an indication of capacity-limited metabolism of PX after its formation from CAF. However, in slow metabolizers capacity-limitedness of PX metabolism might certainly contribute to convexity.

Fits of PX profiles after CAF intake to gamma functions with κ-values left free were very useful in determining the statistical moments of these profiles. In case of convex profiles, numerical methods like the widely used trapezoidal rule are apt to underestimate statistical moments, as Lelo et al. correctly recognized [28]. These authors apparently were ignorant of the possibility of fitting gamma functions to the PX plasma concentration profiles obtained after a single dose of CAF, and escaped from underestimation of AUC by performing their study under steady-state conditions, administering multiple doses of CAF to their subjects.

Combining statistical moments with systems dynamics pharmacokinetics also rendered possible calculation of PX mean body residence time (MRT) after its metabolic formation from CAF. The calculated MRT was applied to correction of the conversion factor $f_M$ of CAF into PX for changing individual disposition rate of the latter compound. The need for such a correction was demonstrated by within-subject variability of values of PX body residence times between experiments in this study. The correction of $f_M$ for changing drug MRT is analogous to the correction of bioavailability for changing drug elimination half-lifes or $t'$s [45-47]. In the present case, MRT was preferred to $t$ as a correction parameter because in the convex PX gamma profiles reflects simultaneous elimination of PX and its formation from CAF.

To our initial surprise, conversion fractions of CAF into PX were significantly higher ($P < 0.05$, pairwise two-tailed $t$-test) after oral intake of CAF than after its intravenous administration. At first we were inclined to attribute the difference to first-pass metabolism of oral CAF. However, the assumption of a notable first-pass effect of ingested CAF is neither supported by literature data, nor by a previous study of our own in which we established virtually complete systemic availability of CAF from coffee and from tea [1b]. Instead, in the latter study evidence was presented for a marked dependence of CAF total clearance on urine flow rate. In a study specifically designed to investigate this dependence, Trang and co-workers [48] explained an observed strongly positive correlation between total clearance of CAF and urine flow rate by suggesting that the primary dimethylxanthine metabolites of CAF might reach sufficiently high concentration levels to competitively retard CAF demethylation, and that increased renal excretion of these dimethylxanthine metabolites at higher urine flow rates could result in a reduction of the competition, thereby increasing metabolic (and total) clearance of CAF.
Urine flow rate of our subjects after oral administration exceeded that after i.v. infusion of CAF (P < 0.01, pairwise 2-tailed t-test), presumably by intake of a water load of 300 ml with the oral dose compared to only 30 ml as an i.v. infusion. Diuresis according to the mechanism proposed by Trang et al. [16] may have resulted in a relatively fast removal of dimethylxanthine metabolites after oral dosing compared to i.v. dosing of CAF, thereby in the former experiment reducing saturation of cytochrome P-450 enzymes mediating demethylation routes in general and the 3-demethylation pathway to PX in particular. In this way, intake of water with oral CAF could have stressed demethylative metabolism of CAF at the expense of other metabolic routes that may not be capacity-limited (figure 1).

Also, increase of renal (and total) clearance of PX due to diuresis following swallowing of a substantial water load with the oral dose may have contributed to the shorter elimination half-life of PX after oral dosage compared to i.v. dosing of PX as such to subject 2.

Studies on the pharmacokinetics of other metabolites of CAF are in progress.

ACKNOWLEDGEMENTS

We are indebted to Dr. A. van Hecken and Inge de Lepeleire for their skillful assistance in experiment performance, to Drs J. Bakker (Dpt of Clinical Pharmacy) for the preparation of the paraxanthine infusion solutions, and to the subjects for their excellent cooperation. The financial support provided by Douwe Egberts Royal Coffee, Tea & Tobacco Factories is gratefully acknowledged.

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Chapter 14

ORAL AND INTRAVENOUS SINGLE DOSE KINETICS OF QUININE
IN HEALTHY MAN

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14 1 SUMMARY

The disposition and absorption kinetics of quinine were investigated in 7 healthy volunteers after intravenous and oral administration. Plasma and urine samples were collected over 45 hours and analyzed for unchanged quinine, and in case of urine samples, also for conjugated quinine, using reversed-phase HPLC combined with fluorescence detection. Pharmacokinetic analysis was done using a non-compartmental dynamic systems approach. Absorption and disposition parameters were calculated from the plasma curves by numerical deconvolution and statistical moments.

Intravenous administration was characterized by a mean residence time of 12.1 h, total clearance of 8.08 l/h, and an apparent volume of distribution of 96.4 l. Renal clearance was 1.26 l/h, and 15.5% of the dose was excreted unchanged in the urine.

After oral administration, median and mean absorption times of quinine were 1.26 and 1.42 h, respectively. Mean systemic availability (± SD) was 0.90 ± 0.18 as calculated by numerical deconvolution, and 0.87 ± 0.11 as estimated by area ratio. In accordance with incomplete availability, mean total urinary recovery (13.4%) was slightly less than that observed after i.v. administration.
tion, while renal clearance and mean residence time were similar. Mean residence times and mean excretion times calculated from plasma and urine data, respectively, correlated well both after oral and iv administration.

In two subjects, dual plasma concentration peaks were seen following oral administration. Conjugated quinine was found in the urine of these subjects, but not in that of the others. On the basis of the available data we could not discriminate with certainty whether the second plasma peaks were due to delayed absorption of quinine after food intake by the subjects, or to enterohepatic circulation of quinine.

Key words Quinine, systems dynamics, pharmacokinetics, numerical deconvolution, mean/median/modal absorption time, systemic availability, enterohepatic recirculation.

Quinine (Q) is one of the oldest drugs in the pharmacopoeia. Until the third decade of this century Q and to a lesser extent the related cinchona alkaloids, represented the sole chemotherapeutic agents for the specific treatment of all four forms of malaria. While synthetic antimalariais largely replaced Q, the emergence of chloroquine-resistant Plasmodial strains in Southeast Asia and South America in the early 1960s, and recently in East Africa, has necessitated its continued therapeutic use.

In the UK Q is widely prescribed as a reliever of nocturnal leg cramps. In a recent double blind, placebo-controlled study, Q failed to effectuate a significant reduction in the number or severity of cramps (Warburton et al 1987).

Q is a marked local irritant. Vascular damage after intravenous administration is the basis for the occasional application of quinine and urea hydrochloride for sclerosing varicose veins and hemorrhoids.

As an oxytocic, Q is daily administered to women during the last 3-4 weeks of pregnancy. With laymen, it enjoys some popularity as an abortifacient. Its effect on the uterus and the developing fetus, however, is highly unreliable.

Q is a common adulterant of illicit heroin according to Berlin et al (1975).

Finally, Q is used as a bitter principal in the flavoring of carbonated soft drinks like 'tonic' or 'bitter lemon', the former drink in the Netherlands containing 40 - 85 mg/l of the alkaloid and the latter less than 40 mg/l.
as laid down in the Dutch Merchandise Legislation. Because of its use in soft drinks, Q has gained the character of a non-prescriptive, mass-consumed luxury drug. Consequently, determination of its kinetics in healthy subjects has gained proportional relevance.

At the same time, detailed comparison of Q kinetics in individuals of different ages and ethnic groups, in health and in various degrees of malaria severity (convalescence, uncomplicated and cerebral malaria), is required to rationalize optimally tailored doses and dosage regimens of this toxic natural product in each subpopulation.

Q kinetics after i.v. administration in caucasian subjects have not been reported previously. Besides, intra-individual comparison of Q kinetics after oral and i.v. administration has not been reported until now. Finally, the analytical method employed in this study is a chromatographical one (HPLC), whereas the majority of earlier investigations of Q kinetics was carried out using the so-called 'double extraction - fluorescence' assay (Cramer & Isaksson 1963), which is thought to quantitate Q metabolites as well to some extent.

14.2 MATERIALS AND METHODS

Subjects Seven volunteers (4 males and 3 females), ranking in age from 21-32 years and in weight from 59-84 kg participated in the study. Written informed consent was obtained according to institutional policy. Volunteers included 2 regular consumers of Q-containing soft drinks, 3 moderate consumers of alcoholics, and one subject taking a bronchodilator. Table I summarizes subject characteristics. Inter alia 60 Hours before the start of an experiment and during its course, subjects refrained from consuming alcohol and Q. They had free access to drinks free of the latter substances, and to food, except in the first three hours after oral administration of Q.

Intravenous quinine administration and sample collection: After collection of a blank blood sample, 150 mg of pyrogen-free Q base (as Q monohydrochloride monohydrate) dissolved in 50 ml normal (0.9%) saline was infused over 12 min. The drug was dispensed with a plastic syringe and an indwelling catheter in the antecubital vein of the left arm. A Braun Perfusor VI pump was used to ensure a constant rate of infusion. A second identical catheter
inserted in the antecubital vein of the opposite arm was used for repeated
blood sampling after removal of the dead space. It was remained patent with
heparinized saline. The subjects remained supine from the start of the infu-
sion until 30 minutes after its termination.

Blood samples were collected in heparinized syringes (5 ml, Monovette®,
Sarstedt) 3, 6 and 12 minutes after starting the infusion, and at 1, 2, 4, 6,
8, 12, 16, 22, 30 minutes and at 1, 2, 5, 9, 22, 30 and 45 hours after its
termination. The infusion catheter was removed immediately at the end of the
infusion, the sampling catheter was removed 1 h afterwards. The remaining
blood samples and the urine samples were taken by individual venipuncture.

During the experimental time, subjects kept a record of time and volume
of each urine voiding. A 100 ml aliquot of each voiding was stored after mea-
surement of the pH. No attempts were made to control urinary pH.

After sampling, plasma was separated by centrifugation at 1000 g for 10
min. Plasma and urine samples were stored at -20°C pending analysis, which
was completed within 5 weeks. No decomposition of Q in spiked plasma or urine was
observed over a 6 months period if stored at the temperature indicated.

Oral quinine administration and sample collection. After collection of a
blank blood sample, a single oral dose of 2.0 mg/kg Q base (as Q sulfate dihy-
drate) was given to each subject in a gelatine capsule at 9:00 a.m. The cap-
sule was ingested with 200 ml of water to prevent sticking in the oesophagus.
Subjects were in the fasting state. A light standard lunch was allowed 3 h
after Q ingestion.

Blood samples were collected by individual venipuncture in heparinized
syringes (10 ml, Monovette®, Sarstedt) from an arm vein at 0:25, 0:5, 0:75, 1,
1:5, 2, 3, 4, 5, 6, 7, 12, 24, 31 and 48 h after ingestion of Q. Again, the
subjects recorded time and volume of urine voidings.

Oral and intravenous administration of Q were performed in a randomized
cross-over design within 1 month for each subject, with one exception. Three
months elapsed between experiments featuring subject 1, who had been adminis-
tered 1.5 mg/kg of Q base per os in an early phase of the program as the oral
pilot subject. Only in this pilot experiment, sample collection was terminated
at 30 h rather than at 45 h.

Plasma and urine quinine analysis. Q in plasma and urine was deter-
mined with a selective and sensitive HPLC technique employing fluorescence
detection as described elsewhere (Teruwen 1988). A 15 cm x 4.2 mm ID reversed-
Phase octadecyl (C18) column was used with 0.08 M acetate buffer (pH = 4.2) and THF (v/v = 60:40) as a mobile phase at a flow of 1 mL/min. Retention times of nonphenolic Q metabolites, Q, and the internal standard methaqualone were 1.9, 2.4, and 4.0 min, respectively.

Coefficients of variation were about 3% for concentrations greater than 0.2 mg/l. Urine samples were analyzed both with and without a preliminary hydrolysis of Q-conjugates by the enzymes β-glucuronidase and arylsulphatase.

Pharmacokinetic Analysis

Intravenous data The plasma concentration-time data \( C_{iv}(t), t \) during the 12 min (T) iv infusion were fitted to the sum of 2 exponentials according to

\[
C_{iv}(t) = \sum (A_i \tau_i / T) (1 - e^{-t/\tau_i})
\]

and after the infusion to

\[
C_{iv}(t) = \sum (A_i \tau_i / T) (1 - e^{-(t-T)/\tau_i})e
\]

From the concentration coefficients \( (A_1, A_2) \) and the time constants \( (\tau_1, \tau_2) \), total area under the plasma concentration vs time curve (AUC) and under the time-concentration versus time curve (TAUC) was computed according to statistical moment theory (Yamaoka et al. 1978)

\[
AUC = \sum A_i \tau_i \quad \text{and} \quad TAUC = \sum A_i \tau_i^2
\]

Furthermore, the AUC until the last collected urine sample (at \( t = Z = 45 \) h), denoted \( AUC_Z \), was approximated as

\[
AUC_Z = AUC - \frac{1}{\tau_2} e^{-Z/\tau_2},
\]

where \( A_2 \) and \( \tau_2 \) represent concentration coefficient and time constant corresponding to the terminal declining part of the plasma concentration versus time curve. The total body transport function \( \psi(t) \) was calculated as

\[
\psi(t) = 1/AUC \sum (A_i \tau_i) (1 - e^{-t/\tau_i})
\]

Mean residence time (MRT), total clearance (CL), and distribution volume \( (V_{dss}) \), and terminal half-life \( (\tau_2) \) were computed as
MRT = TACL/ALC, CL = Dose/ALC, Vdss = MRT*CL, and \( t\frac{1}{2} = 0.693 t\frac{2}{2} \)

Assuming cardiac output CO in our subjects to average 360 l/h, the extraction ratio (E), the average number of recirculations (\( n_{rc} \)) and the mean transit time (MTT) can be calculated as

\[
E = \frac{CL}{CO}, \quad n_{rc} = \frac{(1-E)/E}{1/E}, \quad \text{and} \quad MTT = \frac{MRT}{n_{rc}}
\]

(Van Rossum et al 1983) Renal clearance (CLr) was calculated from CLr = \( Q_r(Z)/AUC_2 \), where \( Q_r(Z) \) is the amount of Q excreted in the urine over 45 h. The cumulative renal excretion, \( Q_r(t) \), was fitted to a sum of two '1 minus' exponentials

\[
Q_r(t) = CL_r \sum_{i=1}^{2} A_i \beta_i (1 - e^{-t/\beta_i})
\]

From the latter \( A_i \) and \( \beta_i \), the mean urinary excretion time (MET) of Q was calculated in analogy to MRT.

**Oral data** The oral concentration curve is a convolution of the absorption function \( H_a(t) \) and the total body transport function \( \psi(t) \) according to

\[
C_{or}(t) = \frac{D}{CL} \ast H_a(t) \ast \psi(t)
\]

where D is the oral dose and '\( \ast \)' denotes the convolution operator. The absorption function \( H_a(t) \) was calculated by deconvolution of the oral plasma data \( (C(t), t) \) and the body transport function \( \psi(t) \) known for each subject from the intravenous experiment, according to the numerical deconvolution method described by Vaughan and Dennis (1978). Statistical moment analysis of the absorption function \( H_a(t) \) yielded the systemic bioavailability (\( h_a \)) and the mean absorption time (MAT)

\[
h_a = AUH_a = \int_0^\infty H_a(t) \, dt, \quad \text{and} \quad \text{MAT} = \frac{TAUH_a}{AUH_a} = \frac{\int_0^\infty t \cdot H_a(t) \, dt}{\int_0^\infty H_a(t) \, dt}
\]

Modal absorption time (ModAT), the time of maximum absorption rate, was estimated as such from the \( H_a(t) \) profile, while median absorption time (MedAT), at which half the available dose is absorbed, was calculated as

260
\[ \int_0^{\infty} H_a(t) \, dt = \frac{1}{2} h_a \]

For a rapidly absorbed drug like Q sulfate \( H_a(t) \) need not be calculated \textit{ad infinitum}. In practice, the upper integrand 't' may be replaced by the time at which the oral plasma curve attains its terminal phase of decline.

The oral \((C_{or}(t), t)\) data were also fitted to a triexponential equation

\[ C_{or}(t) = \sum_{i=1}^{2} A_i \left( e^{-t/\tau_i} - e^{-t/\tau_0} \right) \]

In fitting the double-peaked curves of subjects 1 and 4, two doses were assumed, the quantity and the dosage time of the 2nd dose were iteratively optimized. Again AUC, AUC\(_Z\) and TAUC were calculated, and from these

\[ \text{MRT} = \text{TAC}/\text{AUC} - \text{MAT}, \quad \text{and} \quad \text{CL}_r = Q_r(Z)/\text{AUC}_Z \]

Cumulative renal excretion, \( Q_r(t) \), was fitted to a sum of three '1 minus' exponentials

\[ Q_r(t) = \text{CL}_r \left[ \sum_{i=1}^{2} A_i \left( \tau_i \left( e^{-t/\tau_i} - 1 \right) - \tau_0 \left( e^{-t/\tau_0} - 1 \right) \right) \right] \]

Again, from the \( A_i \) and \( \tau_i \) obtained in the latter fit, mean urinary excretion time (MET) of Q was calculated in analogy to MRT.

### 14.3 RESULTS

**Intravenous dosing** The plasma concentration vs time plot following intravenous infusion of 150 mg of Q in a representative subject, and the Q transport function \( \psi(t) \) in this subject are shown in figure 1 (left). Following an initial rapid decrease in the plasma Q concentration \((\tau_1 = 3.56 \pm 0.69 \text{ min})\), the terminal declining part of the curve was characterized by mean (± SD) time constants of \( \tau_2 = 12.25 \pm 1.82 \text{ h} \), corresponding to terminal elimination half-lives of \( 8.49 \pm 1.26 \text{ h} \). The Q transport function (or unit impulse response function) \( \psi(t) \) may be viewed as a frequency distribution of residence times and therefore is proportional to the plasma concentration profile after i.v. injection of Q (Van Rossum et al. 1983). Time constants and corresponding
FIGURE 1. (Top) Plasma concentration profiles of quinine in subject 7 following (left) i.v. infusion of 150 mg over 12 min, (right) ingestion of a gelatine capsule containing quinine sulphate. (Bottom left) Total body transport function $\psi(t)$ of quinine in this subject (displayed on two time scales) ...
FIGURE 1 (cont'd). ... as calculated from the i.v. profile. (Bottom right) Gastro-intestinal absorption rate of quinine vs time in subject 7 following capsule ingestion. The absorption profile was calculated from the 'oral' concentration vs time curve and $\psi(t)$ by numerical deconvolution.
<table>
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<th>A_2</th>
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</table>

**MEAN** 25.69 6.12 1.55 3.56 12.3 19.0 18.5 233 ± SD 4.9 3.66 0.29 0.69 1.8 3.3 3.1 61

**BW** = body weight, **Med/Ind** = medication and drugs of indulgence, **V** = user of **Ventolin** (salbutamol), **Q** = regular drinker of soft drinks containing quinine, **A** = modest consumer of alcohol (± 10 g/day)

### TABLE II PHARMACOKINETIC PARAMETERS OF QUININE AS CALCULATED FROM THE PLASMA CONCENTRATION VS TIME PROFILE DURING AND FOLLOWING INTRAVENOUS INFUSION

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<th>V_{dss}</th>
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<th>CL_r</th>
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</table>

**MEAN** 8.5 12 8.08 96 2.3 16 45 23.6 1.26 11.6 5.9 60 ± SD 1.3 1.8 1.36 18 0.4 3.8 5.4 0.18 2.0 0.2 22

For calculation of E, MTT, and N_{rc}, the subjects' cardiac output C O was taken 360 l/h.

\[ t_f = \ln 2 \cdot \tau_2 = 0.693 \cdot \tau_2, \text{ } pH = \text{mean urinary pH}, \text{ } UFR = \text{mean urine flow rate} \]
concentration coefficients for the individual subjects have been listed in Table I, together with the derived statistical moments AUC, (AUC\textsubscript{2}) and TAUC.

The pharmacokinetic parameters calculated from the fitted data are given in Table II. The mean residence time was about 12.0 h. Total clearance of Q averaged 8.09 ± 1.36 l/h and its mean apparent volume of distribution amounted to 96.5 ± 17.7 l (mean ± SD). During each circulation transit through the body, a fraction E = 2.3 ± 0.4% of Q molecules is extracted by biotransformation and excretion. On the average, a Q molecule is able to make N\textsubscript{RI} = 45 body transits before being eliminated one way or the other, transits lasting 16.1 ± 2.9 min (= MTT) on the average.

Following the infusion, 15.5 ± 3.9% of the Q dose was recovered unchanged in subjects' urine over a period of 45 h. The cumulative renal excretion curve of subject 3 is shown in figure 3. Renal clearance amounts to 1.26 ± 0.18 l/h and is independent of plasma Q concentration in the range of concentrations attained during the experiments) as indicated by linearity of Q\textsubscript{R}(t) versus AUC\textsubscript{R} plots in all subjects, where t are times of urine voiding. Urinary recovery for none of the subjects was elevated discernably following hydrolytic enzym treatment of urines prior to analysis. Mean urinary excretion times of Q in each subject correlated reasonably with mean residence times in plasma (MRT = 11.6 ± 2.0 h, mean ± SD).

Oral dosing The plasma concentration-time profile following ingestion of Q sulfate in a gelatine capsule by subject 7, and the absorption rate versus time profile in this subject are shown in figure 1 (right). Q is rapidly absorbed, mean absorption times ranging from 1.2 h (mean ± SD 1.42 ± 0.34 h). Averaged median and modal absorption times are virtually equal (1.26 h) and slightly smaller than the MAT, indicating slight tailing of absorption. Systemic availability of Q averaged (± SD) 0.90 ± 0.18 (range 63 - 113 %). Individual absorption times are listed in Table III.

Oral dosing The plasma concentration-time profile following ingestion of Q sulfate in a gelatine capsule by subject 7, and the absorption rate versus time profile in this subject are shown in figure 1 (right). Q is rapidly absorbed, mean absorption times ranging from 1.2 h (mean ± SD 1.42 ± 0.34 h). Averaged median and modal absorption times are virtually equal (1.26 h) and slightly smaller than the MAT, indicating slight tailing of absorption. Systemic availability of Q averaged (± SD) 0.90 ± 0.18 (range 63 - 113 %). Individual absorption times are listed in Table III.

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Mean residence time, mean urinary excretion time, and renal clearance after oral administration were almost identical to the values observed after i.v. dosing (Table III). In accordance with incomplete bio-availability, total urinary excretion of unchanged Q was somewhat less after ingestion (13.4 ± 4.0 % of dose, mean ± SD) than after i.v. dosing. The cumulative renal excretion of Q in subject 3 after oral dosing is included in figure 3. In five of the subjects, urinary recovery of Q was not changed by more than 1% after enzymatic hydrolysis of urine specimens. However, 45 h-pooled urine of subjects 1 and

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TABLE III. CURVE FITTING DATA AND PHARMACOKINETIC PARAMETERS OF QUININE AFTER ORAL ADMINISTRATION OF 2.0 MG/KG\(^{\circ}\) OF QUININE IN A GELATINE CAPSULE

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<th>AUC(_Z)</th>
<th>TAUC</th>
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<td>h</td>
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<td>1.8</td>
<td>0.3</td>
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\(pH = \text{mean urinary } pH, \text{ UFR = mean urine flow rate}\)

\(\circ\) Subject 1 was administered 1.5 mg/kg of oral quinine (oral pilot study).

* MAT, MedAT, ModAT, and \(h_a\) for subjects 1 and 4 apply to the first absorption peak. The other parameters listed for these two subjects were not corrected for secondary absorption, as estimation of the amount of drug involved in secondary absorption was considered inaccurate (because of lack of sufficient plasma concentration data in the time interval of secondary absorption), and because this amount was small anyway (≤ 12% of the oral dose).

4 showed 5.4 and 4.7 mg increments of Q content (5.0 and 3.5% of dose) respectively, after hydrolysis of Q conjugates.

In these same two subjects, a second plasma concentration peak was observed approximately 6 h after oral dosing. The dual-peaked plasma concentration - and the absorption rate - versus time profiles for subject 1 is shown in figure 2. The amounts of Q giving rise to the second peak in plasma concentration profiles, and hence in the absorption rate profiles of subjects 1 and 4, came to 11.2 % and 5.6% of oral dose, respectively.
FIGURE 2 Time profiles of (top) plasma concentration and (bottom) gastrointestinal absorption rate of quinine in subject 1 after swallowing quinine sulphate in a capsule. Note the dual peaks in both profiles, and the analogy of this figure to the right part of figure 1.
Few pharmacokinetic reports deal in a detailed way with Q kinetics after iv administration (White et al 1982, White et al 1983a) or after oral administration (Berlin et al 1975, Wanamolruk et al 1986) to healthy adults or patients convalescing after an attack of falciparum malaria. While largely confirming the results of these earlier studies, new information with respect to bioavailability, disposition and excretion of Q is provided by the intra-individual comparison of Q pharmacokinetics after its administration by iv infusion and per os as the most widely used preparation, the sulfate salt in a gelatin capsule, and by the employment of chromatographical analysis in this study, rather than a double extraction (Cramer and Isaksson 1963) or protein precipitation assay (Brodie et al 1943), both of which are thought to
quantitate Q metabolites in various degrees (Trenholme et al. 1976, Edstein et al. 1983, White 1985)

**Distribution and disposition** The only pharmacokinetic report of iv Q administration to healthy man is the publication by White et al. (1983a) who gave 5 mg/kg of Q over a 5 min infusion to 7 male Thai subjects. In the present study in caucasian subjects, a mean dose of 2.2 mg/kg was infused over 12 min.

In both studies a biexponential decay of plasma Q was observed after termination of the infusion, characterized by time constants of 'distribution' and 'elimination' phases of a few minutes, and many hours, respectively. Mean terminal half-life and apparent volume of distribution of Q observed in White's subjects both are somewhat larger than in the present subjects (11.1 vs. 8.5 h, and 1.8 vs. 1.4 l/kg, resp.), whereas mean total clearances were essentially the same (115 vs. 117 ml/h/kg). See Tables I and II. Berlin et al. (1975) observed increasing individual half-lives and distribution volumes with increasing Q dose, clearance remaining constant. Thus, discrepancies in the two former kinetic parameters in White's study (1983a) and the present one could reflect a dose dependence of Q kinetics. However, calculation of distribution volume and, hence, total clearance as performed by Berlin et al. is highly questionable, and dose dependence of Q kinetics remains to be confirmed in other studies. In particular, Q pharmacokinetics were similar after 10 and 20 mg/kg initial doses to patients with cerebral malaria (White et al. 1983b). Plasma Q levels in the latter study (10-15 mg/l) significantly exceeded those produced by Berlin and colleagues (1975) in their volunteers (<10 mg/l).

Sex, genetics influences, and differences in environmental factors such as diet, smoking, and habitual consumption of alcohol and xanthine beverages, might as well underly the observed differences in Q half-life and distribution volume of our subjects and those of White et al. (1983a). Differences in kinetic parameters between patients convalescing from malaria (White et al. 1982) and the normal volunteers in this study in addition may result from malaria-induced changes of Q kinetics (e.g., as a result from altered plasma protein binding of Q) Silamut et al. 1985 that may still be apparent in these patients to a certain extent.

**Absorption and availability** Q sulfate is the most frequently used oral Q preparation (White 1985). The present study has shown that in healthy subjects oral Q, formulated as a powder in a gelatine capsule, becomes available
rather rapidly (MAT = 1.4 h) and rather completely (averaged 0.90) Extent of availability is comparable to the study of Hall et al. (1973), in which oral administration of Q sulfate in gelatine capsules produced plasma Q levels and amounts of excreted Q that were 10-20% lower than corresponding figures obtained after 1 h infusion of Q Similar results were obtained by Wanwimolruk and co-workers (1986) who (over-) estimated total clearance of oral Q sulfate as (oral dose)/AUC and obtained a mean figure of 10.0 l/h as compared to 8.1 l/h in the present study Greenblatt et al. (1977), and Conrad et al. (1977) found the availability of oral quinidine sulfate to be 0.81 (range 0.61-1.08) and 0.87 (range 0.62-1.20), respectively. Q and quinidine are stereoisomers (diastereomers), which on the basis of their almost identical physico-chemical properties (Notterman et al. 1986) may be hypothesized to have similar absorption kinetics. As the relatively low soluble Q sulfate was found to be 90% bioavailable, at least equally complete availability may be anticipated for equivalent formulations of more soluble Q salts like the di- and monohydrochloride, and for aqueous solutions (or soft drinks) of Q Plain and sugar-coated tablets of these Q salts appear to be less well absorbed (Garnham et al. 1976)

Incompleteness of systemic availability of oral Q preparations may as well in part be due to first-pass metabolism of these drugs. Computing hepatic Q clearance ($CL_m\text{ }^{\text{m}}$) as the difference of total and renal clearance of Q, and estimating hepatic blood flow ($\dot{V}_{\text{hep}}$) as 90 l/h in our healthy subjects, the quotient $CL_m/\dot{V}_{\text{hep}}$ in case of linear first-pass kinetics represents the first-pass extraction ratio, averaging $7\frac{1}{2}$% in our subjects.

Data on the rapidness of GI absorption of Q are scarce. Wanwimolruk et al. (1986) recorded peak plasma levels of Q to occur 1.5 h after ingestion of the sulfate salts. Ochs et al. (1980) collected and summarized literature data on quinidine absorption after oral administration. In ten different studies using quinidine sulfate tablets, and two additional ones concerned with a capsule and an aqueous solution of this salt, peak plasma levels were perceived at 1.0 - 3.1 h (mosty 1½-2 h) after dosage. Reported absorption half-lifes varied from 14 to 38 min.

While these data at first sight compare fairly well to the absorption times listed in Table III, it should be remarked that, firstly, time intervals elapsing between dosing and plasma peaks provide no insight into the course of the absorption process, and secondly, that the specification of first-order absorption half-lifes ($t_{\frac{1}{2}}$) or rate constants ($k_a$) implicates the assumption of a (mono-) exponential decline of absorption rate with time.
Considering the complex concerted action of fluctuating factors affecting absorption rate, such as dissolution rate of solid drug formulations, rate of gastric emptying, intestinal peristaltics, pH-gradient, and drug dispersion to, and consequent simultaneous absorption from various intestinal sites at different rates, it is obvious that the assumption of first-order absorption kinetics in general is an oversimplification which can only serve as a crude first approximation of the absorption process. Limiting ourselves to Q and quinidine, our argumentation is supported by the frequently noticed bumpy appearance of plasma concentration-time profiles after oral dosing of these drugs (present study; Greenblatt et al. 1977; Salvadori et al. 1982; Sawyer et al. 1982). Moreover, absorption profiles derived from 'smooth' oral curves, as obtained for subjects 2, 3, and 5-7 in the present study (for subject 7, see figure 1c/d), neither show evidence of exponential decline, though the profiles slightly tail as indicated by the somewhat larger MAT compared to MedAT (Table III).

Calculation of the absorption rate profile by numerical deconvolution of the oral plasma curve and the drug's transport function is generally applicable as it assumes only linearity of kinetics and time-invariability of drug distribution and disposition. Fickleness of absorption, the rule rather than the exception, thus is left in its own right. Only the ascending leg and the peak(s) of the oral plasma curve is needed for calculation of the absorption rate profile $H_a(t)$. As soon as the oral curve comes up to its terminal declining part, absorption in general has stopped; the remainder of oral concentration data are irrelevant for $H_a(t)$ computation. For drugs having a mean residence time that is great compared with the mean absorption time of a particular formulation of this drug (as in the present case: $12.2 \text{ h} = \text{MRT} > > \text{MAT} = 1.4 \text{ h}$), the time interval in which absorption takes place constitutes only a minor fraction of total time of bloodsampling. In this condition, early cutting off oral data at twice or thrice the MAT renders the calculated absorbed amount of drug highly independent of intra-individual variability in disposition kinetics. Such variability has been observed for caffeine (Teeuwen 1987) and for many other drugs (Rowland 1980) and may seriously affect calculations of absorbed drug fractions using 'whole curves' as does Dost's widely used law of corresponding areas (AUC ratio method): $h_a = \frac{\text{AUC}_{\text{or}}}{\text{AUC}_{\text{iv}}} \times \frac{D_{\text{iv}}}{D_{\text{or}}}$. As $Q$ elimination kinetics in the present study were individually invariable, the AUC ratio method should yield a similar estimate of systemic availability of $Q$ after oral dosage. It did: $h_a = 0.87 \pm 0.11$. 271
Absorption times of subject 6 were greater than in any of the other subjects, though the differences just failed to reach significance (for MAT and MedAT, $P = 0.1$ as judged by Dixon's test) Subject 6 at the time of the experiments happened to be under clinical observation because of vague digestive disturbances recurring irregularly in fits persisting for some weeks. These disturbances eventually were diagnosed as sprue. As in this disease absorptive area is reduced by atrophy of intestinal villi, retardation of absorption in this subject is comprehensible. Extent of absorption nevertheless was virtually complete in this subject.

Conditions that may impair GI absorption in complicated falciparum malaria include vomiting, jejunitis, and intestinal lesion associated with acute falciparum malaria (Olsson & Johnston 1969, Karney & Tong 1972). As both rate and extent of $Q$ absorption may be significantly reduced, in these conditions $Q$ infusion is the preferred choice of administration.

**Renal excretion** After iv administration of $Q$, $15.5 \pm 3.9\%$ of the dose was excreted unchanged in our subjects' urine, whereas, consistent with incomplete availability, after oral dosing only $13.4 \pm 4.0\%$ was recovered. Excreted fractions in the present study largely confirm the findings of other investigators, though being rather small Hall et al (1973) and White et al (1982) recovered 19 and 22\% of intravenous $Q$ in urine voided by convalescing malaria patients, while Hall et al (1973) and Brooks et al (1969) came across 15 and 17\% of oral $Q$ sulfate (as tablets) in their convalescing patients' urine on the average.

As the period of urine collection in the present experiments must be considered long enough for excretion to be virtually complete ($\text{AUC}_2/\text{AUC} \geq 0.94$), genetic and environmental factors or disease-altered kinetics as mentioned before could underly the differences in excreted fractions. Alternatively, the extraction-fluorescence assay employed by the cited authors may co-quantitate $Q$-metabolites that are separated from $Q$ in the HPLC assay employed in this study. Significantly, when comparing their HPLC assay with extraction-fluorescence, Edstein et al (1983) observed 37% higher plasma 'quinine' levels using the latter method.

Mean urinary pH and flow did not differ between oral and iv experiments (cf Tables II and III). Renal clearance did not differ either and averaged $1.26 \ell/h$ which is close to the value calculated by Notterman et al (1986) on the basis of HPLC analytical results ($1.3 \ell/h$). As $Q$ in healthy individuals is bound to plasma proteins for some 90\% (Silamut et al 1985, 272
renal clearance of unbound Q ranges from 10 to 15 l/h in our subjects, probably exceeding glomerular filtration rate in each of them. It is concluded that tubular secretion contributes to renal Q clearance as established previously by Notterman et al. (1986).

Systemic availability of oral Q as estimated from excreted percentages of Q after oral and i.v. administration amounts to 0.86 ± 0.11 and is comparable to the availability estimates from plasma data either by numerical deconvolution or by AUC ratio method.

Multiple plasma peaks, food intake, and enterohpatic circulation. Multiple plasma concentration peaks like those observed in subjects 1 and 4 some 6-8 h after oral administration of Q were also observed by Salvadori et al. (1982) in their one pilot subject, and by Sawyer (1982), Greenblatt (1977) and their respective co-workers in part of their subjects after oral administration of quinidine. Subjects in these studies and the present one were sober until 3-4 h after oral administration, and then were allowed to have lunch. Renewed absorption of drug left behind in the GI tract hence could result from stimulation of intestinal motility after food intake. This interpretation of dual peak pattern implicates that true systemic availability of Q for subjects 1 and 4 should be augmented by the amounts absorbed in second instance, yielding \( h_a = 0.84 \) and \( h_a = 0.90 \) respectively (cf. Table III). Importantly, multiple peaks have never been observed after intravenous administration of Q or quinidine to our knowledge.

However, the coincidence of dual plasma peaks and urinary excretion of notable amounts of conjugated Q in the same two subjects suggests some kind of relationship between these phenomena. The link could be enterohpatic circulation of Q in subjects 1 and 4, as suggested by Sawyer et al. (1982) to effect dual plasma peaks of quinidine in two of their subjects. Food intake again might play a triggering role, by increasing bile production and biliary secretion of Q, whether or not in conjugated form. Unfortunately, Sawyer et al. (1982) and Greenblatt et al. (1977) did not perform urine analysis. Salvadori et al. (1982) did, and recovered 20% more Q in their subject's urine with a preliminary enzymatic hydrolysis than without one. This figure compares fairly well to the 29 and 23% elevations of Q recovery in the 45 hours pooled urine of our subjects 1 and 4, respectively, due to enzymatic pretreatment.

If dual peaks in these subjects result from enterohepatic circulation, then their absence after i.v. administration may implicate that first-pass metabolism by gut wall or liver is related to the enterohepatic cyclus. As
remarked earlier, about $7\frac{1}{2}$ % of absorbed Q may be extracted in during the first liver passage. The amount of Q accumulated in the liver during the first pass may persist for some hours, and may then be biliary secreted in bulk after food intake by the subject, be re-absorbed, and give rise to a pronounced secondary peak. This intriguing matter requires future attention.

ACKNOWLEDGEMENTS

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14 5 REFERENCES


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APPENDIX I. SYNTHESIS OF 4',4'-DIDEUTERO COTININE

The synthetic route to 4',4'-dideutero cotinine consisted of eight steps:

Step 1 An amount of 184 ml (2.5 Moles) of SOCl₂ was added drop by drop to 123 g (1 Mole) of nicotinic acid. The mixture was reflux-boiled for 2.5 h. The excess of SOCl₂ was evaporated. The resulting light-yellow colored solid was dissolved in a mixture of 160 ml (2 Moles) of pyridine and 100 ml of CH₂Cl₂. While stirring, a solution of 85 g (1 Mole) of pyrrolidone in 50 ml of CH₂Cl₂ was added in drops, followed by reflux-boiling (15 min) and stirring (19 h, ambient temperature) of the reaction mixture. Subsequently a 500 ml solution of NaHCO₃ (1.1 Moles) was added and the reaction product was extracted with CH₂Cl₂. The extract was dried over MgSO₄. Then the CH₂Cl₂ was distilled off to yield a black solid, which was recrystallized three times in ethyl acetate to produce light-yellow colored needles of N-nicotinoyl-2-pyrrolidinone (m.p. = 102-103°C). The yield was 56 g (29 %).

Step 2. 39 g (205 mMoles) of N-nicotinoyl-2-pyrrolidinone and 31 g (205 mMoles) of ethyl nicotinate were dissolved in 300 ml of dry THF. The solution was heated to 70°C and added in drops to a suspension of 5.28 g (220 mMoles) of NaH in 100 ml of dry THF (75°C). The reaction mixture was kept under an argon atmosphere for 60 h at 70°C and then was cooled to 0°C by addition of ice and pH-neutralized with concentrated HCl. Extraction with CH₂Cl₂ and evaporation of this solvent after drying over MgSO₄ gave 1,3-dinicotinoyl-2-pyrrolidinone. Yield: 45 g (74 %).

Step 3. 45 g (153 mMoles) of crude 1,3-dinicotinoyl-pyrrolidone was dissolved in 200 ml of concentrated HCl (37 %). The solution was reflux-boiled until no more carbon dioxide evolved (approximately 2½ h). The solution was cooled to 0°C and rendered slightly alkaline by addition of an appropriate amount of K₂CO₃. The reaction product was subsequently extracted with CHCl₃ and dried over MgSO₄. The CHCl₃ was evaporated to produce 10 g of a dark-brown oily residue, which was purified by bulb to bulb distillation (Kugelrohr) at 160°C and 4 Torr to yield 8.5 g (38 %) of the white solid myosmine as confirmed by the 1H-NMR spectrum of the reaction product.
Step 4  The glassware used in this step was dried in an oven at 110° and 100 Torr. 8.5 g (58 mMoles) of myosmine were dissolved in 50 mL of D$_2$O (99.75 %), and 3 g of K$_2$CO$_3$ (dried overnight at 150° and 100 Torr) was added. The solution was stirred for 5 days at 85°. Then the reaction product was CHCl$_3$-extracted, dried, and concentrated. The yield was 6.5 g of 3',3'-dideutero-myosmine (76 %).

Step 5  6.5 g (44 mMoles) of 3',3'-dideutero-myosmine was dissolved in 500 mL of an ethanol-water mixture (1:3, v/v). 3.34 g (88 mMoles) of NaBH$_4$ was added and the suspension was stirred for 7 days at ambient temperature. The reaction product was extracted with five 150 mL-portions of CHCl$_3$, dried over MgSO$_4$, and the solvent was removed by distillation. Yield 6.15 g (93 %) of 3',3'-dideutero-nornicotine, a light-yellow colored oil.

Step 6  6.15 g (41 mMoles) of 3',3'-dideutero-nornicotine and 6.8 g (68 mMoles) of N(CH$_2$CH$_3$)$_3$ was dissolved in 200 mL of diethyl ether. A solution of 4.47 g (41 mMoles) of ClCOOCH$_2$CH$_3$ in another 100 mL of diethyl ether was slowly added while stirring. After 5 min the reaction mixture was filtrated and the filtrate concentrated by ether evaporation. The residue consisted of 8.4 g (92 %) of (N-ethoxy-carbonyl)-4',4'-dideutero-nornicotine.

Step 7  8.4 g (38 mMoles) of (N-ethoxy-carbonyl)-3',3'-dideutero-nornicotine was dissolved in 100 mL of dry THF and added drop by drop to a well-stirred solution of 3 g (80 mMoles) of LiAlH$_4$ in another 200 mL of dry THF. The reaction mixture was stirred for 50 h at ambient temperature. Thereafter it was cooled to 0°, and 5 mL of aqua dest., 3 mL of a 15 % NaOH-solution, and another 5 mL of aqua dest., respectively, were added. Subsequently the reaction mixture was filtrated, the filtrate was extracted with diethyl ether, washed with two 5 mL-portions of aqua dest., dried over MgSO$_4$, and concentrated. The yield was 5.8 g of a light-yellowish oil, which was purified by bulb to bulb distillation (Kugel rohr) (84°, 0.5 Torr) to yield 3.4 g (55 %) of a colorless oily liquid 3',3'-dideutero-nicotine. 1 g of the latter compound was stored as such and hence was not subjected to the final step.

Step 8  To a well-stirred solution of 2.4 g (14.6 mMoles) of 3',3'-dideutero-nicotin in 10 mL of acetic acid (80 %) a solution of 5 mL of
bromine in another 15 ml of acetic acid (80 %) was added in drops The reac-
tion mixture was diluted with a 75 ml aliquot of distilled water and heated to
80° until the deuterated nicotine was dissolved Then it was cooled slowly to
4° and stirred for 16 h The solid reaction product (3',3'-dideutero-nicotine
perbromide) was suspended in 50 ml of 5 N HCl 11 g of zinc powder was added
gradually in the course of 3.5 h The suspension was stirred for one more
hour Then the pH of the suspension was set to 8 - 9 with ammonia The reac-
tion product was extracted with four 150 ml-portions of CH₂Cl₂ and dried over
MgSO₄ The CH₂Cl₂ was distilled off to yield 1.4 g (54 %) of the colorless
oily-appearing 4',4'-dideutero-cotinine (cotinine-D₂), which was stored in an
inert argon atmosphere The overall reaction yield of the eight successive
synthesis steps was approximately 1 %

Purity checks The thus synthetized cotinine-D₂ was found to be ≥ 99% chemi-
cally pure by spectrometrical (UV, IR, and ¹H-NMR) and chromatographica-
spectrometrical techniques (reversed-phase HPLC with UV-monitoring at 254 nm,
GC-NSD, GC-MS)

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execution of the actual synthesis
APPENDIX II THE LAPLACE TRANSFORM

Definition of the Laplace Transform and its Inverse

The Laplace transform is defined in the following manner. Let \( F(t) \) be a real function of a real variable \( t \) defined for \( t > 0 \). Then

\[
\mathcal{L}\{F(t)\} = \mathcal{F}(s) \equiv \lim_{\varepsilon \to 0^+} \int_{\varepsilon}^{\infty} F(t)e^{-st}dt, \quad \text{for } 0 < \varepsilon < \infty
\]

is called the Laplace transform of \( F(t) \). \( s \) is a complex variable defined by \( s = \sigma + j\omega \), where \( \sigma \) and \( \omega \) are real variables and \( j \) is the basic imaginary number, equal to \( \sqrt{-1} \).

Note that the lower limit on the integral is \( t = \varepsilon > 0 \). This definition of the lower limit is sometimes useful in dealing with functions which are discontinuous at \( t = 0 \). The real variable \( t \) always denotes \textit{time}.

If \( F(t) \) is defined and single-valued for \( t > 0 \) and \( F(\sigma) \) is absolutely convergent for some real number \( \sigma_0 \), that is,

\[
\lim_{\varepsilon \to 0^+} \int_0^{\infty} |F(t)|e^{-\sigma t}dt < \infty, \quad \text{for } 0 < \varepsilon < \infty
\]

then \( F(t) \) is Laplace transformable for \( \text{Re}(s) = \sigma > \sigma_0 \).

The Laplace transforms a problem from the real variable time domain into the complex variable \( s \) domain. After a solution of the transformed problem has been obtained in terms of \( s \), it is necessary to 'invert' this transform in order to obtain the time domain solution. The transformation from the \( s \) domain into the \( t \) domain is called the \textit{inverse Laplace transform}. Let \( F(s) \) be the Laplace transform of a function \( F(t), t > 0 \). The contour integral

\[
\mathcal{L}^{-1}\{F(s)\} = F(t) = \frac{1}{2\pi j} \int_{c-i\infty}^{c+i\infty} F(s)e^{st}ds
\]

where \( j = \sqrt{-1} \) and \( c > \sigma_0 \) is called the \textit{inverse Laplace transform} of \( F(s) \).

The following properties of the Laplace transform and its inverse, and the table of Laplace transforms given in this appendix may help to circumvent the actual performance of contour integrations in many instances.
Some properties of the Laplace transform and its inverse

- The Laplace transform is linear. That is, if $F(s)$ and $G(s)$ are the Laplace transforms of $F(t)$ and $G(t)$, respectively, then $\mathcal{L}(fF(t) + gG(t)) = fF(s) + gG(s)$, where $f$ and $g$ are arbitrary constants.

- The inverse Laplace transform is linear. That is, if $F(t)$ and $G(t)$ are the inverse Laplace transforms of $F(s)$ and $G(s)$, respectively, then $\mathcal{L}^{-1}(fF(s) + gG(s)) = fF(t) + gG(t)$, where $f$ and $g$ are arbitrary constants.

- The Laplace transform of the derivative $dF/dt$ of a function $F(t)$ whose Laplace transform is $F(s)$ is
  \[ \mathcal{L}\left(\frac{dF}{dt}\right) = sF(s) - \lim_{t \to 0} F(t) \]

- The Laplace transform of the integral $\int_0^t F(t)dt$ of a function $F(t)$ whose Laplace transform is $F(s)$ is
  \[ \mathcal{L}\left(\int_0^t F(t)dt\right) = \frac{F(s)}{s} \]

- The initial value $F(0)$ of the function $F(t)$ whose Laplace transform is $F(s)$ is
  \[ F(0) = \lim_{t \to 0} F(t) = \lim_{s \to \infty} sF(s) \quad \text{for } t > 0 \]

  This relation is called the Initial Value Theorem.

- The final value $F(\infty)$ of the function $F(t)$ whose Laplace transform is $F(s)$ is
  \[ F(\infty) = \lim_{t \to \infty} F(t) = \lim_{s \to 0} sF(s) \quad \text{for } t > 0 \]

  If $\lim_{t \to \infty} F(t)$ exists, this relation is called the Final Value Theorem.

- The Laplace transform of a function $F(t/a)$ (Time Scaling) is
  \[ \mathcal{L}(F(t/a)) = aF(as) \]

  where $F(s) = \mathcal{L}(F(t))$. 
• The inverse Laplace transform of a function \( F(s/a) \) (Frequency Scaling) is

\[
\mathcal{L}^{-1}\{ F(s/a) \} = aF(at)
\]

where \( \mathcal{L}^{-1}\{ F(s) \} = F(t) \).

• The Laplace transform of the function \( F(t-T) \) (Time Delay) where \( T > 0 \) and \( F(t-T) = 0 \) for \( t \leq T \), is

\[
\mathcal{L}\{ F(t-T) \} = e^{-aT}F(s)
\]

where \( F(s) = \mathcal{L}\{ F(t) \} \).

• The Laplace transform of the function \( e^{-at}F(t) \) (Complex Translation) is given by

\[
\mathcal{L}\{ e^{-at}F(t) \} = F(s+a)
\]

where \( F(s) = \mathcal{L}\{ F(t) \} \).

• The Laplace transform of the product of two functions \( F(t) \) and \( G(t) \) is given by the complex convolution integral

\[
\mathcal{L}\{ F(t)G(t) \} = \frac{1}{2\pi i} \int_{c-i \infty}^{c+i \infty} F(\omega)G(s-\omega)d\omega
\]

where \( F(s) = \mathcal{L}\{ F(t) \} \), \( G(s) = \mathcal{L}\{ G(t) \} \).

• The inverse Laplace transform of the product of two transforms \( F(s) \) and \( G(s) \) is given by the convolution integrals

\[
\mathcal{L}^{-1}\{ F(s)G(s) \} = \int_0^t F(\tau)G(t-\tau)d\tau = \int_0^t F(t-\tau)G(\tau)d\tau = F(t)*G(t)
\]

where \( \mathcal{L}^{-1}\{ F(s) \} = F(t) \), \( \mathcal{L}^{-1}\{ G(s) \} = G(t) \).
Some Laplace Transform Pairs useful for Dynamical Systems Analysis in Pharmacokinetics

<table>
<thead>
<tr>
<th>Functions in time domain</th>
<th>Laplace transforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F(t)$ $(t \geq 0)$</td>
<td>$F(s)$ $(= \mathcal{L}(F(t))$</td>
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<tr>
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<tr>
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<td>$a/s$</td>
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<tr>
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<tr>
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<td>$+ (z-c)e^{-ct}/(a-c)(b-c)$</td>
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</tr>
<tr>
<td>$e^{-at}\sin(\omega t)$</td>
<td>$\omega/(s+a)^2+\omega^2$</td>
</tr>
<tr>
<td>$\cos(\omega t)$</td>
<td>$s/(s^2+\omega^2)$</td>
</tr>
<tr>
<td>$e^{-at}\cos(\omega t)$</td>
<td>$(s+a)/(s+a)^2+\omega^2$</td>
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<tr>
<td>$-{(dc^2e+cf)/(b-c)(c-a)}e^{-ct}$</td>
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### Functions in time domain

\[ F(t) \quad (t \geq 0) \]

1. \( \frac{1}{a^2} (1 - e^{-at}) \)
2. \( \frac{1}{a^2} (z - z e^{-at} + a(a-z)t e^{-at}) \)
3. \( e^{-at} (c + (b-ca)t) \)
4. \( \frac{1}{a^2} (at-1+e^{-at}) \)
5. \( \frac{d}{ab} \left( \frac{(a^2-ca+d)/(a(b-a))}{e^{-at}} + \frac{(b^2-cb+d)/(b(b-a))}{e^{-bt}} \right) \)
6. \( t^n \)
7. \( t^{n-1} / (n-1)! \quad 0! = 1 \)
8. \( t^{n-1} e^{-at} / (n-1)! \)
9. \( (t/a)^k e^{-t/a} \quad k=0,1,2,3,... \)
10. \( (t/a)^k e^{-t/a} \quad k \ll 1, \infty \)

### Laplace transforms (continued)

\[ F(s) \quad (= \mathcal{L}(F(t)) \]

1. \( \frac{1}{s(s+a)^2} \)
2. \( \frac{(s+z)/(s(s+a)^2)}{(s+\gamma)/(s(s+a)^2)} \)
3. \( \frac{1}{(s(s+a)^2)} \)
4. \( \frac{1}{(s^2+cs+d)/(s(s+a)(s+b))} \)
5. \( \frac{1}{s^2} \)
6. \( n!/s^{n+1} \quad \text{unit ramp} \)
7. \( 1/s^n \quad \text{polynomial} \)
8. \( (s+a)^{-n} \quad n=1,2,3,... \)
9. \( k!/(a^k(s+1/a)^{k+1}) \quad \text{'gamma function'} \)
10. \( \Gamma(k+1)/(a^k(s+1/a)^{k+1}) \quad \text{'gamma function'} \)
This dissertation deals with the human pharmacokinetics of three alkaloids of plant origin on the boundary of medical and luxury drugs, which are being produced in huge quantities in the developing countries, and being consumed worldwide but especially so in western society. Despite the mass consumption and the immemorial utilitive history of nicotine, caffeine and quinine, there still exist considerable gaps of knowledge with respect to the pharmacokinetics and -dynamics of these drugs. The research work described in the present dissertation was aimed at bridging some of these gaps.

The investigations are based upon a systems dynamics approach of pharmacokinetics. As this modern approach is not (yet) generally accepted in this field, SECTION I, Chapter 1 as well, presents an introduction into systems dynamics pharmacokinetics.

Firstly the characteristics of an isolated organ or tissue regarding the passage of a quantity of drug injected at the arterial site are described. A well-stirred flow vessel appears to be a reasonably good model for a single organ/tissue. The most important features of such a single organ are the magnitude of the blood flow through it, as well as the mean, median and modal transit times of the injected molecules.

Subsequently, bearing in mind physiology and anatomy of the human body, the total body (or system) is composed of the various organs and tissues (the subsystems) in an imaginary construction process. The most prominent feature of the system is the blood circulation, which feeds drug output back as input in a proportional way and transforms the body into a so-called positive feedback system. The dynamics of the body system are governed by those of the subsystems, and may conveniently be summarized into the total body transport function \( \psi(t) \). \( \psi(t) \) in essence is the probability density distribution of drug molecule residence times in the body, and differs for each drug and individual. Even intra-individually, \( \psi(t) \) is a function of numerous (patho-)physiological processes, such as cardiac output, disease, and age.

Mathematically the concentration vs. time profile of a drug in plasma (the drug-output) is the convolution-integral of the body transport function \( \psi(t) \) and the rate at which the drug molecules reach the circulation (the drug-input). The latter in turn depends on dosage regimen, formulation, and route.
of administration of the drug. The affection of drug input rate by formulation and route of administration may be condensed in the application transport function.

Be two of the three functions input-, transport-, and output function known, then the third unknown function can be computed from the former two by either convolution or deconvolution. The body transport function $\psi(t)$ of a drug may be derived from the plasma concentration vs time profile (output) after an accurately known drug input (e.g., by intravenous injection or constant intravenous infusion) in a subject or patient. Once $\psi(t)$ has been determined, the drug output concentration in this person for an intended input may be predicted by convolution and, if so desired, be adjusted by adjustment of the input. Also, for any given output and $\psi(t)$ the input function can be calculated back by deconvolution. This is an excellent technique to determine bioavailability of drugs reaching systemic circulation by absorption from an application site, and to elucidate rate and extent of conversion of a parent drug into a particular metabolite.

For a precise knowledge of empirical input- en output data, accurate analytical methods are indispensable. In SECTION II the assays employed in the presently reported investigations have been allocated. Except for a single working-up technique all methods were newly developed, and have in common a liquid or gas-liquid chromatographical separation procedure.

In Chapter 2 a capillary gaschromatographical method combined with nitrogen-detection is proposed for the estimation of nicotine and its principal metabolite cotinine in biological fluids. An important advantage in favour of this method is the simultaneous occurrence of sample preparation and chromatographical separation for nicotine and cotinine. The assay moreover is very rapid, which predominantly is due to the potent single-step solid phase extraction sample work-up. Although applicable to saliva and urine, this method was employed mainly to analyze nicotine and cotinine in blood plasma of smoking experimental volunteers.

The smoked-out cigarette filters collected by these volunteers were analyzed for nicotine using an assay described in Chapter 3. The filter preparation technique involving crushing and ultrasonic vibration was kindly passed on to us by the Department of Analytical Chemistry of the Netherlands Organization for Applied Scientific Research (TNO).
Chapter 4 deals with a bioassay, derived from the GC-NSD method in Chapter 2, in which the nitrogen detector has been replaced by a mass spectrometer with the facility of 'selective ion monitoring' (GC-MS-SIM). This assay enabled us to detect cotinine and a cold-isotope labelled (di-deuterated) cotinine-analogue separately in smokers' plasmas. The smokers were given perorally a small dose of the labelled cotinine (cotinine-D2) at one of the two days of laboratory attendance. The administration was followed by complete absorption and served the purpose of determining total cotinine clearance in each smoking volunteer.

Chapter 5 describes a gaschromatographical technique which utilizes a short packed column and nitrogen detection to estimate caffeine levels in caffeine-containing drinks and in plasma en saliva of human volunteers. The chromatographical separation (runtime 3 min) is the most rapid ever reported, with one exception. This method was put into service in the preliminary investigations preceding the human kinetic caffeine trials, to gain insight in the dose of caffeine to be expected in a given volume of freshly brewed coffee and tea, and to analyze body fluids of kinetic pilot volunteers.

In the time of the above pilot experiments a liquid chromatographic method for the analysis of caffeine in beverages and body fluids became available. This method using UV-detection at 254 nm is reported in Chapter 6 and is also suited to co-monitor the 3 dimethylxanthine metabolites of caffeine. Furthermore the liquid chromatograph was equipped with an automated injection facility. Because of these advantages, the HPLC method (actually a composition of closely related methods with extraction conditions, constitution of mobile phase, and choice of internal standard varying with the nature of the sample, the separative potency of the column, and the identity of the analyte(s)) came to replace the earlier developed GC method in the remainder of the caffeine studies.

Eventually Chapter 7 presents a HPLC-method combined with fluorescence detection, suited for the analysis of quinine concentrations in infusion solutions, blood plasma and urine of experimental volunteers.

In the last section, SECTION III, the results of the clinical-pharmacokinetical investigations of nicotine, caffeine, and quinine are described.

Chapter 8 deals with the methodology and the results of an investigation of the nicotine intake by smokers of conventional (i.e., unventilated) filter-
tipped cigarettes Habitual and inhaling smokers of 4 cigarette brands belonging to the conventional class (coded Fl t/m F4) were recruited to constitute 6-membered populations of each brand (with the exception of the smokers' population of brand F4, which consisted of 7 volunteers). The smokers collected their butts during a 16-day period and in this period provided blood samples while attending the laboratory for two days separated by a one-week interval. Smoking machine studies revealed nicotine retention in the cigarette filters of the 4 brands studied to be about one third, this relative retention largely being independent of smoking technique. This characteristic rendered the cigarette filter a valuable tool to estimate smokers' nicotine intake at mouth-level. Butts of human-smoked filter cigarettes were analyzed for (absolute retention of) nicotine, whereas blood plasma in addition was analyzed for nicotine-derived cotinine and cotinine-D2. The latter compound was given orally in a gelatine capsule to the volunteers. The results of filter and plasma analyses were combined to resolve the intake issue.

In general the intake of nicotine intra-individually varied only slightly between the 2 days of laboratory attendance. Inter-individual fluctuations of nicotine intake per cigarette between the smokers of a single brand on the other hand were considerable and amounted to about a factor 2 for all brands. The amount of nicotine taken in at mouth-level by the smokers of the respective brands as determined by filter analysis was (mean ± SD, mg of nicotine per cigarette): 1.58 ± 0.48 mg for Fl, 1.05 ± 0.16 mg for F2, 1.51 ± 0.42 mg for F3, and 1.67 ± 0.48 mg in case of F4. The nicotine imprints as determined by machine smoking of cigarettes of these brands amounts to 1.2 mg for Fl, 1.1 mg for both F2 and F3, and 1.0 mg for F4.

The majority (a fraction \( f_L \)) of the nicotine reaching the oral cavity of the volunteers was absorbed in the pulmonary circulation, and for the major part (a fraction \( f_M \)) converted into the main metabolite cotinine. The product of the fractions \( f_L \) en \( f_M \) as averaged over the smokers of all 4 brands (± SD): \( f_L \cdot f_M = 0.66 ± 0.10 \). For a degree of conversion of \( f_M = 0.75 \), the systemic (blood-level) intake of nicotine was computed, which amounted to (mean ± SD, mg of nicotine per cigarette): 1.34 ± 0.34 mg for the smokers of Fl, 0.96 ± 0.20 mg for those of F2, 1.27 ± 0.28 mg for the smokers of F3, and 1.31 ± 0.22 mg for those of F4.

We conclude that the nicotine label as printed on the packet is a reasonably good indication of the mean intake of smokers of the studied brands. The large inter-individual variability of the intake is in favour of introducing nicotine packet imprints as classes (intervals) rather than as a single number as is custom nowadays.
In Chapter 9 the results of an identically designed investigation of nicotine intake by habitual smokers of 2 ventilated cigarette brands, coded as VI (6 smokers) and V2 (4 smokers) are reported. The 'ventilatedness' of these brands refers to the presence of minute perforations in the cigarette filter, through which extraneous air may be introduced during a puff. In this way the tobacco smoke may be diluted, resulting in relatively small nicotine (and tar) yields as determined by machine smoking of cigarettes of this class, as shown by the nicotine labels of brands VI (0.2 mg) en V2 (0.1 mg). Human smokers may smoke with a degree of ventilation less than maximum, and the relative nicotine retention in the filters of these cigarettes is a function of the degree of ventilation with which they are smoked. Therefore, the amount of nicotine reaching the oral cavity of the smoker for a measured absolute nicotine filter retention cannot be estimated as accurately as for conventional filter cigarettes. In practice, the accuracy of the estimation of this amount appeared to be amply sufficient.

The nicotine intake per cigarette at mouth-level amounted to 1.67 ± 0.46 mg for the smokers of VI and to 0.73 ± 0.25 mg for those of V2 on the average (± SD). The product of the systemic availability $f_L$ of inhaled nicotine and its fractional conversion $f_M$ into cotinine was 0.68 ± 0.10 for the smokers of VI and V2. For $f_M = 0.75$ the systemic nicotine intake of the smokers was determined and amounted to 1.43 ± 0.27 mg for those of VI, and 0.69 ± 0.24 mg for those of V2. Both mouth-level and systemic intake of nicotine by the smokers of VI and V2 are significantly greater than the machinally determined packet imprint. In fact, the systemic intake did not approach the packet imprint closer than a factor 5 to 6 for any of the volunteers. Of all 6 conventional and ventilated brands studied, VI delivered the greatest amount, and V2 the smallest amount of nicotine to habitual smokers of each brand.

The difference between the nicotine imprint on the packet and intake of nicotine by human smokers must be due to corresponding differences in smoking technique between the smoking machine and the human smokers. The observed differences are too large to be accounted for by assuming that human smokers puff more frequently or with larger puff volumes than the smoking machine does. The latter assumption may very well be true, but it is also almost inevitable to co-assume that our volunteers have blocked the ventilation mechanism of their cigarettes wholly or in part with their fingers or lips. Despite the small number of smokers of VI en V2 involved in the study, the observed tendency is evident to such an extent that the value of the standardized machine smoking
procedure to predict the intake of nicotine (and probably of the other constituents of the aerosol phase of cigarette smoke) in general must be considered as limited.

In Chapter 10 the intake of carbon monoxide (CO) by the former smokers of conventional and ventilated cigarettes is discussed. The study design involved the analysis of CO in smokers' exhaled alveolar air just before and just after smoking single cigarettes. The increase of the CO-concentration due to smoking was converted to the \textit{in vivo} CO-yield of the cigarette with the aid of additional data (body weight, blood hemoglobin concentration).

Conventional and ventilated filter cigarettes were indistinguishable with respect to their CO-delivery to smokers, which varied from 14 to 20 mg of CO per cigarette as averaged over the smokers' populations of each of the 6 brands. Within the population of a single brand, average individual CO-intakes amounted to a factor 2 - 5 between highest and lowest exposure. Even larger differences existed between the CO-yields of single cigarettes subsequently smoked by the same volunteer. No relationship was found between the individual average intake of nicotine on the one hand and that of CO on the other by the volunteers. We did find a significant and negative correlation between the estimated total amount of hemoglobin in the volunteers' bodies and the average increment of the alveolar air CO concentration after cigarette smoking. Existence and sign of this correlation are intelligible regarding the linear relation between CO-concentrations in breath and in blood, where CO occurs almost totally as a CO-hemoglobin complex.

Because women dispose of fewer hemoglobin than men on the average, but (in this study) take in just as much as CO as their male fellow-smokers, women build up higher blood CO concentrations and hence in theory are more apt to experience the toxic effects of CO than men.

Chapter 11 deals with the pharmacokinetics of caffeine (CAF) following intravenous infusion of a 200 mg dose over 30 min in 6 young and healthy volunteers, equally distributed over the sexes. The transport function $\psi(t)$ of CAF was monoexponential for one subject and biexponential for the 5 others, the terminal exponent outweighing the rapid initial exponent in terms of statistical moments. The mean ($\pm$ SD) time constant $\tau_2$ of the terminal exponent was $6.7 \pm 2.0$ h ($= \tau_{2\text{,ave}} = 4.7 \pm 1.4$ h). A conspicuously large time constant ($\tau_2 = 9.9$ h) was observed in a female taking oral contraceptives, whereas a very short $\tau_2$ (4.2 h) was found in a stiff smoker. Owing to the dominance of the terminal
exponent the mean residence time (± SD) of CAF in the body (6.5 ± 2.0 uur) approximates \( \tau_2 \), and the variance of body residence times (46 uur\(^2\)) is about the square of the mean residence time.

Mean ± SD of the plasma clearance and the apparent volume of distribution of CAF in our volunteers was 7.2 ± 3.6 l/h and 42 ± 8 l, respectively. Volume of distribution of CAF approximately corresponds to total body water.

Each circulatory transit a fraction (the extraction ratio of CAF) of 2.0 ± 1.0% of circulating CAF molecules was eliminated. The circulatory transit time, and the number of transits completed by a CAF molecule before being eliminated, amounted to 7.0 ± 1.3 min and 56 ± 17, respectively, as averaged over molecules and subjects.

The same volunteers who were administered CAF by intravenous infusion were given coffee and tea on two other occasions, as described in Chapter 12. CAF present in both these beverages was absorbed virtually completely from the GI tract (for 99 ± 4% in case of coffee, and for 95 ± 6% in case of tea). Absorption was also rapid; the median absorption time (at which half the available quantity is absorbed) was for CAF drunk as coffee 20 ± 3 min, and for CAF as tea 19 ± 3 min. Mean absorption time was slightly higher due to 'tailing' of the absorption process 26 ± 5 min for CAF as coffee, and 23 ± 3 min for CAF as tea. Differences between coffee and tea with regard to rate and extent of CAF absorption were small and not significant. Absorption rate vs time profiles showed a fickle multi-peaked course for each of the volunteers.

Individual volumes of CAF distribution were rather constant between the 3 experiments involving CAF administration as i.v. infusion, coffee, and tea, whereas total clearance (CL) and the time constant of elimination (\( \tau_{el} \)) displayed substantial intra-individual fluctuations between experiments. It was inferred that the elimination kinetics of CAF in our subjects very likely was dose dependent (non-linear). In the tea treatment, involving by far the smallest CAF dose administered, CI and \( \tau_{el} \) were significantly greater and smaller, respectively, than in the coffee and intravenous CAF treatments.

Comparison of CL en \( \tau_{el} \) in the two latter treatments yielded the conclusion that the disposition rate of CAF in our volunteers also depended strongly upon their urine flow, which after dosage of coffee (and of tea) was significantly higher than following the i.v. infusion (in part owing to the aqueous volume of the coffee/tea itself). This dependence on urine flow has its origins in the proposed dose dependence of CAF disposition rate. As renal clearance of CAF itself is negligible in adults, the linking of CAF-disposition to urine flow...
flow probably is established by way of the 3 dimethylxanthines, into which CAF is converted for ± 95% in phase I of its metabolism.

The dimethylxanthines compete with CAF for the one or multiple enzymes of cytochrome P-450, which effect demethylation of xanthines, and at the same time undergo renal clearance to an important extent. Increase of urinary flow accelerates renal elimination of the dimethylxanthines, reduces competition of CAF with these compounds for P-450, and thus results in a higher total clearance and a shorter elimination time constant of CAF.

In Chapter 13 the kinetics of CAF are compared to those of its main metabolite paraxanthine (1,7-dimethyl xanthine, PX) in the 3 male volunteers having participated in the above CAF-experiments. The total body transport function $\Psi(t)$ of PX was monoexponential in two volunteers and biexponential in the third, the terminal exponent outweighing the initial one in terms of statistical moments. Pharmacokinetical parameters of CAF and PX differed only moderately. The time constant of PX elimination $\tau_2$ amounted to 4.6 ± 0.9 h on the average (± SD) (cf. 5.9 ± 1.9 h for CAF). Plasma clearance and volume of distribution of PX assumed mean values (± SD) of 11.8 ± 2.2 l/h and 52 ± 5 l (cf. 8.9 ± 4.8 l/h, and 45 ± 9 l for CAF).

After oral administration in aqueous solution PX was absorbed rapidly (mean absorption time 22 ± 1 min) and virtually completely (for 94 ± 10%, cf. 23 ± 7 min, and 103 ± 13% for CAF, respectively).

The similarity of elimination time constants of parent CAF and metabolic daughter PX effects a typically convex plasma PX concentration profile after a single dose of CAF, which profile often cannot be described adequately by a sum of exponentials, but all so much the better by a so-called gamma function.

Of an intravenous dose of CAF 77 ± 7% was converted into PX by the 3 volunteers, whereas this percentage was 84 ± 9% (mean ± SD) for an equal oral dose of CAF. Since a 'first-pass' effect for CAF has never been reported in literature, the cause of the difference in fractional conversion of oral and i.v. doses of CAF to PX probably is due to the difference in urine flow of the volunteers. The latter was importantly higher after after oral than after i.v. dosage of CAF, partly owing to the large water load co-administered with the oral CAF dose. Probably the high urinary flow rate in the oral experiment effected a relatively rapid (renal) excretion of PX and other dimethylxanthines generated from CAF by demethylation. Hence competition between CAF and the dimethylxanthines for the demethylating enzymes was reduced, speeding up CAF disposition rate along the demthylation route (see Ch. 12), and stressing this metabolic route at the cost of other routes.
Eventually in Chapter 14 quinine kinetics in 7 young and healthy subjects (4 males, 3 females) are discussed both after intravenous infusion and after oral administration of a gelatine capsule containing the quinine sulphate salt. The body transport function \( \psi(t) \) was biexponential in all volunteers. The time constants of both exponents diverged widely and amounted to \( 3.6 \pm 0.7 \) min, and \( 12.3 \pm 1.8 \) h (mean \( \pm \) SD). In terms of statistical moments the terminal exponent was dominant by far. The mean body residence time of quinine \( (12.0 \pm 1.8 \) h) therefore closely approximated \( \tau_2 \). Total quinine clearance in the subjects amounted to \( 81 \pm 14 \) l/h, and the distribution volume to \( 96 \pm 18 \) l.

Averaged over volunteers and molecules, quinine molecules took \( 16 \pm 3 \) min to complete one circulatory transit in the body, and completed a total of \( 45 \pm 8 \) transits before being eliminated from the circulation. Per transit a fraction (the extraction ratio) of \( 2.3 \pm 0.4 \% \) of quinine molecules was eliminated, a minority of which (about 16% on the average) was excreted unchanged in the urine. Renal clearance of quinine amounted to about \( 1.3 \pm 0.2 \) l/h, suggesting tubular secretion of quinine, since the plasma-protein-bound quinine fraction is as great as 90%.

Following oral administration of quinine sulphate in a gelatine capsule quinine was absorbed to a high extent \( (90 \pm 18 \%) \) from the GI tract. The absorption rate vs time profile of oral quinine was regular and symmetrical as indicated by the close proximity of median, modal, and mean absorption time \( (1.25 \pm 0.38 \) h, \( 1.26 \pm 0.41 \) h, and \( 1.42 \pm 0.35 \) h). Absorption was slowest in a volunteer who afterwards was shown to suffer from sprue.

In two volunteers considerable time after the first absorption peak a second absorption phase was seen, in which an additional \( 11\% \) en 6% of the oral quinine dose was absorbed. Since this second phase occurred just after the subjects' lunch, food intake constitutes a possible explanation for the renewed occurrence of absorption. However, the presence of small quantities of the glucuronide conjugate of quinine in urine voidings of exclusively these very same two volunteers would indicate enterohepatic recirculation of quinine as the phenomenon underlying the occurrence of dual absorption peaks. In this case food intake again could be the triggering event which gets the recirculation going.

Because urinary quinine glucuronide was found only after oral, but not after i.v. administration of quinine in the two volunteers mentioned, quinine conjugation might be related to the so-called 'first pass.' On the basis of
the available data the question of the dual absorption peaks could not be definitely resolved.
Samenvatting en Conclusies

Dit proefschrift behandelt de humane farmacokinetiek van drie plantaardige alkaloiden op het grensvlak van genot- en geneesmiddel, die in enorme hoeveelheden in de ontwikkelingslanden worden geproduceerd en mondiaal, maar vooral in de Westerse samenleving, worden geconsumeerd. Ondanks hun massale verbruik en niettegenstaande de eeuwenoude gebruikshistorie van deze drie stoffen, die ons terugvoert tot de dageraad van de mensheid, bestaan er nog altijd aanzienlijke hiaten in de wetenschappelijke kennis van farmacokinetiek en -dynamiek van deze stoffen. Dit proefschrift poogt enkele van deze hiaten te dichten.

Het beschreven onderzoek is geschoeid op de leest van de systeemdynamische benadering van de farmacokinetiek. Daar deze moderne benadering op dit wetenschapsgebied (nog) geen gemeengoed is, geeft SECTIE I, tevens Hoofdstuk 1, een inleiding in de materie.

Eerst passeren de eigenschappen van een geïsoleerd orgaan of weefsel met betrekking tot de doorstroomsnelheid van een aan de arteriële kant aangeboden hoeveelheid farmacon de revue. Het blijkt dat een goed geroerd doorstroomvat een redelijk model is voor een enkel orgaan. De belangrijkste kenmerken van het geïsoleerde orgaan zijn de bloedstroom en de gemiddelde transit-tijd die de farmaconmoleculen nodig hebben om het orgaan te passeren.

Vervolgens wordt, rekening houdend met fysio- en anatomische feitelijkheden, het totale menselijk lichaam (het systeem) in een denkbeeldig constructieproces opgebouwd uit de afzonderlijke organen en weefsels (de subsystemen). Het meest kenmerkende van het systeem is de bloedcirculatie, die farmacon-output evenredig terugvoert als -input en van het lichaam een zgn. positief feedback systeem maakt. De dynamiek van het totale systeem wordt bepaald door die van de subsystemen, en kan worden samengevat in de totale lichaamstransportfunctie \( \psi(t) \).
Dichtheidverdeling van verblijftijden van farmaconmoleculen in het lichaam, en is voor elke stof en elk individu anders, en is ook voor een gegeven persoon en stof een functie van tal van (patho-)fysiologische processen, zoals hartminutenvolume, ziekte, en ouderdom.

Mathematisch gezien is het concentratieprofiel van een farmacon in bloed(-plasma) (de farmacon-output) de convolutie-integraal van de lichaamstransportfunctie \( \psi(t) \) en de snelheid waarmee dat farmacon de bloedbaan bereikt (de farmacon-input). Dit laatste hangt op zijn beurt weer af van doseringsschema, formulering, en toedieningsweg van dat farmacon. De invloed van formulering en toedieningsweg op de farmacon-input kan worden uitgedrukt in een applicatie-transportfunctie.

Zijn van de drie functies input-, transport-, en outputfunctie, er twee bekend, dan kan de derde uit deze twee worden berekend door rekentechnieken die bekend staan als convolutie en deconvolutie. De lichaamstransportfunctie \( \psi(t) \) van een farmacon is te berekenen uit het concentratieprofiel (output) na een nauwkeurig bekende input (bv. intraveneuze injectie of constant infuus van de stof) in een proefpersoon of patiënt. Is \( \psi(t) \) eenmaal bekend, dan kan voor deze persoon het concentratieprofiel bij een voorgenomen input worden voor-speld en eventueel bijgesteld (convolutie). Ook kan bij gegeven output en \( \psi(t) \) de inputfunctie worden teruggerekend door deconvolutie. Dit is een uitmuntende techniek ter bepaling van de biologische beschikbaarheid van farmaca die via een absorptiefase de systemische circulatie bereiken, en ter bepaling van de snelheid en de mate waarmee een verbinding wordt omgezet in een bepaalde metaboliet.

Voor een nauwkeurige kennis van experimentele input- en outputgegevens zijn goede analysemethodieken een noodzaak. In SECTIE II zijn de in het thans beschreven onderzoekswerk gebruikte analytische methoden verzameld. Op een onderdeel na zijn de methoden alle speciaal voor dit onderzoek ontwikkeld, en hebben een vloeistof- of gaschromatografische scheidingsprocedure met elkaar gemeen.

In Hoofdstuk 2 wordt een capillair gaschromatografische methode met stik-stofdetectie beschreven voor de bepaling van nicotine en diens voornaamste metaboliet cotinine in biologische vloeistoffen. Een belangrijk voordeel van deze methode boven eerder ontwikkelde en gepubliceerde methoden is dat monstervoorbewerking, scheidings en detectie van nicotine en cotinine simultaan plaatsvinden. De methode is bovendien erg snel, hetgeen voor een belangrijk
deel te wijten is aan de enkel-staps, zeer krachtige voorbewerkingsmethode waarbij gebruik wordt gemaakt van een vaste-fase extractietechniek met wegs-}
werpkolommetjes. Met deze methode werden nicotine en cotinine in het bloed-
plasma van proefpersoon-rokers geanalyseerd.

De berookte sigarettenfilters van deze proefpersonen werden op nicotine geanalyseerd met de analysemethode beschreven in Hoofdstuk 3, waarvan de voorbewerkingswijze ons vriendelijk werd toegespeeld door de afdeling Analytische Chemie van TNO.

Hoofdstuk 4 behandelt een techniek, afgeleid van de GC-NSD methode in hoofdstuk 2, waarbij de stikstofdetector is vervangen door een massaspectrometer met 'selective ion monitoring'-faciliteit (GC-MS-SIM). Deze techniek stelde ons in staat de nicotine-metaboliet cotinine en een koud isotoop-gelabeld cotinine-analoog separaat te detecteren in het plasma van de proefpersoon-rokers. De proefpersonen kregen een kleine dosis van het gelabelde cotinine (4',4'-dideutero cotinine) perorale toegediend op een van de twee dagen van hun aanwezigheid in het laboratorium. Op deze wijze kon een basale farmacokinetische parameter van cotinine (totale plasmaklaring) tijdens het experiment bepaald worden met minimale beïnvloeding van het rookgedrag.

Hoofdstuk 5 beschrijft een gaschromatografische techniek die gebruikmaakt van een korte gestapelde kolom en stikstofdetectie om caffèine te bepalen in caffèine-houdende dranken en in plasma en speeksel van menselijke vrijwilligers na toediening van caffèine. De chromatografische scheiding is de op een na snelste ooit gerapporteerd (runtime 3 min). Deze methode werd ingezet in de voorbereiding op de caffèine-studies, waarbij de te verwachten caffèine-dosis in koffie en thee werd ingeschat, en in de farmacokinetische 'pilot'-experiments.

Ten tijde van de bovengenoemde 'pilot'-experiments kwam een vloeistofchro-
matografische methode voor caffèine gereed voor gebruik. De HPLC methode met UV-detectie, beschreven in Hoofdstuk 6, had als voordelen boven de eerder ontwikkelde GC-methode dat naast caffèine zelf de drie dimethylxanthine metabolieten van caffèine mede konden worden geanalyseerd, en dat gebruik kon worden gemaakt van een automatische injectiefaciliteit. De HPLC methode (eigenlijk een samenstel van nauw verwante methoden, waarbij extractiemilieu, samenstelling van de mobiele fase en keuze van interne standaard wisselden af naar gelang de aard van het monster, de kwaliteit van de HPLC-kolom en de te bepalen substraten) werd voor het vervolg van het caffèine-project gebruikt ter analyse van caffèine en de dimethylxanthines in koffie, thee, infusievloeistoffen, en lichaamsvloeistoffen.
Tenslotte wordt in hoofdstuk 7 een HPLC-methode met fluorescentie-detectie beschreven, geschikt voor het meten van de concentratie van kinine in infusievloeistoffen, bloedplasma en urine van proefpersonen gemeten werd.

In SECTIE III worden de resultaten van het klinisch farmacokinetisch onderzoek aan nicotine, caffeine, en kinine uit de doeken gedaan.

Hoofdstuk 8 beschrijft de methodologie en de resultaten van een onderzoek naar de nicotine-inname van rokers van conventionele (dus ongeventileerde) filtersigaretten. Van 4 sigarettemerken (gecodeerd als F1 t/m F4) uit deze klasse werden vrijwilligerspanels samengesteld, elk bestaande uit 6 gewoongetrouwde inhalerende rokers van deze merken (het panel van merk F2 bestond als enige uit 7 rokers). De vrijwilligers verzamelden gedurende een 16-daagse periode hun sigarettepeukcn en waren in deze periode tevens 2 dagen te gast op het laboratorium, waar bloedmonsters werden afgenomen. Door middel van rookmachine-studies aan de 4 onderzochte sigarettemerken kwam vast te staan dat ongeveer 1/3 deel van de aan het sigarettefilter aangeboden hoeveelheid nicotine in het filter wordt achtergehouden, ongeacht de rooktechniek. Deze eigenschap maakt van het sigarettefilter een waardevol hulpmiddel ter schatting van de hoeveelheid nicotine die de mondholte van de roker bereikt. De peukcn van door proefpersonen gerookte sigaretten werden geanalyseerd op nicotine, het bloedplasma bovendien op cotinine en cotinine-D2 welke laatste stof de vrijwilligers oraal kregen toegediend. De analyseresultaten van filters en plasma werden gecombineerd ter oplossing van het innamevraagstuk.

In het algemeen variëerde de inname van nicotine bij elke vrijwilliger slechts weinig tussen de 2 experimentele dagen. Tussen de vrijwilligers in elk van de panels bleken echter aanzienlijke verschillen van de nicotine-inname per sigaret te bestaan, voor drie van de merken signaleerden we een factor 2 discrepantie tussen de zwaarste en de lichtste roker in het panel. De nicotine-inname op mondniveau bedroeg voor de rokers van de merken (gemiddelde ± SD, mg nicotine per sigaret) 1.58 ± 0.48 mg voor F1, 1.05 ± 0.16 mg voor F2, 1.51 ± 0.42 mg voor F3, en 1.67 ± 0.48 mg voor F4. De pakjes-vermelding van nicotine, zoals die door machinaal verroken wordt vastgesteld, bedraagt 1.2 mg voor F1, 1.1 mg voor F2 en F3, en 1.0 mg voor F4.

Van de hoeveelheid nicotine die de mondholte van onze rokers bereikte werd het merendeel (een fractie fL) in de bloedbaan opgenomen, en daarvan weer het merendeel (een fractie fM) in de metaboliet cotinine omgezet. Het product van de fracties fL en fM bedroeg gemiddeld over de rokers van alle merken (± SD) 1x.
0.66 ± 0.10 Voor een omzettingsgraad $f_M = 0.75$ werd de *systemische* inhalatie van nicotine berekend. Deze bedroeg per sigaret (gemiddeld ± SD) 1.34 ± 0.34 mg voor F1, 0.96 ± 0.20 mg voor F2, 1.27 ± 0.28 mg voor F3, en 1.31 ± 0.22 mg voor F4.

We concluderen dat het nicotine-getal op het pakje een redelijke indicatie is voor de inhalatie van de gemiddelde roker van de onderzochte merken. De grote inter-individuele variatie van de inhalatie pleit echter voor de opgave van een nicotine-klasse of -interval in plaats van het tot dusverre gebruikelijke enkelvoudige getal.

In Hoofdstuk 9 passeren de resultaten van een onderzoek naar de nicotine-inname van rokers van 2 merken geventileerde filtersigaretten, gecodeerd als V1 (6 rokers) en V2 (4 rokers) de revue. Opzet en uitvoering van het onderzoek waren identiek aan die van het onderzoek aan conventionele filtersigaretten. Het 'geventileerd' zijn van de filtersigaretten refereert aan de aanwezigheid van minuscule perforaties in het filter, waardoor tijdens een trek lucht aangezogen kan worden, waarmee de tabaksrook wordt verdund. De verdunning van de rook leidt bij machinaal verroken van sigaretten uit deze klasse tot spectaculair lage nicotine (en teer) opbrengsten, zoals de nicotinecijfers op de pakjes van V1 (0.2 mg) en V2 (0.1 mg) aantonen. Bij menselijk roken kan de ventilatiegraad gereduceerd worden door blokken van het ventilatiemechanisme. De relative retentie van nicotine in de filters van deze sigaretten is afhankelijk van de ventilatiegraad, waardoor bij gemeten absolute retentie de hoeveelheid nicotine die de mondholte van de roker bereikt, minder nauwkeurig kan worden berekend dan voor conventionele filtersigaretten. In de praktijk blijkt de accuratesse van de schatting van deze hoeveelheid toch ruimschoots voldoende.

De nicotine-inname per sigaret op mond-niveau bedroeg gemiddeld (± SD) 1.67 ± 0.46 mg voor V1 en 0.73 ± 0.25 mg voor V2. Het product van de systemische beschikbaarheid $f_L$ van geïnhaleerd nicotine en de omzettingsgraad $f_M$ in nicotine was 0.68 ± 0.10 in de rokers van V1 en V2. Voor $f_M = 0.75$ werd de *systemische* nicotine-inname van deze rokers berekend 1.43 ± 0.27 mg voor rokers van V1, en 0.69 ± 0.24 mg voor die van V2. Deze cijfers zijn voor beide merken statistisch significant hoger dan het door de rookmachine bepaalde pakjesvermelding. De systemische inname benaderde de pakjesvermelding voor geen enkele van de vrijwilligers dichter dan een factor 5 à 6. Van alle zes onderzochte merken leverde V1 per sigaret de grootste, en V2 de kleinste hoeveelheid nicotine aan de panelrokers.
Het verschil tussen pakjesvermelding en menselijke inname van nicotine moet gezocht worden in corresponderende verschillen in rooktechniek tussen rookmachine en menselijke rokers. De gevonden verschillen zijn te groot om alleen verklaard te worden door aan te nemen dat de rokers in de panels hun sigaretten met meer en grotere trekken verroken dan de rookmachine dat doet. Dit kan zeer wel het geval zijn, maar daarnaast moet haast wel worden aangenomen dat deze rokers het ventilatiemechanisme van hun sigaret gedeeltelijk dan wel geheel blokkeren met vingers en/of lippen. Ondanks het kleine aantal rokers van V1 en V2 in de studie is de gevonden trend zo duidelijk aanwezig, dat de voorspellende waarde van de gestandaardiseerde machinale rookprocedure voor de inname van nicotine (en waarschijnlijk ook de andere bestanddelen van sigaretterook) door rokers van geventileerde filtersigaretten in het algemeen gering moet worden geacht. Een aanpassing van genoemde procedure voor geventileerde sigaretten is derhalve gewenst.

In Hoofdstuk 10 wordt de inname van koolmonoxide door de (zelfde) rokers van de conventionele en geventileerde sigaretten behandeld. De opzet van de studie voorzag hier in het analyseren van koolmonoxide (CO) in de adem van de rokers juist voor en juist na het roken van individuele sigaretten. Het increment in de CO-concentratie werd m b v. additionele gegevens (lichaamsgewicht, hemoglobinegehalte in bloed) omgerekend naar de in vivo CO-opbrengst van die sigaret.

Conventionele en geventileerde filtersigaretten waren ononderscheidelijk met betrekking tot CO-dosis die aan de rokers werd geleverd, en die gemiddeld over de rookpanels uiteenliep van 14 - 20 mg CO per sigaret. De verschillen tussen de rokers van hetzelfde merk waren groter dan voor nicotine, en beliepen een factor 2 1/2 - 5 tussen de zwaarste en de lichtste roker in de diverse panels. Ook de verschillen in CO-opbrengst van opeenvolgende sigaretten, gerookt door eenzelfde individu, waren aanzienlijk. Tussen de hoogte van gemiddelde inname van nicotine enerzijds en CO anderzijds van onze vrijwilligers bestond geen verband. Wel werd een significante en negatieve correlatie gevonden tussen de geschatte totale hoeveelheid hemoglobine in het lichaam van de roker en de grootte van de concentratiesprong van CO in adem voor en na het roken van een sigaret. De correlatie is begrijpelijk gezien het lineaire verband tussen de CO-concentraties in adem en in bloed, waar CO geheel aan hemoglobine gebonden voorkomt. Aangezien vrouwen gemiddeld over minder hemoglobine beschikken dan mannen, maar (althans in deze studie) evenveel CO binnenkrijgen door roken, bouwen zij hogere bloedconcentraties aan CO op dan mannen en zullen zij in theorie de toxische effecten eerder ondervinden.
Hoofdstuk 11 behandelt de farmacokinetiek van caffeine (CAF) na intraverneuze infusie van een dosis van 200 mg over 30 min in 6 jonge gezonde vrijwilligers, gelijkliefk verdie over beide geslachten. De transportfunctie $\psi(t)$ van CAF was van monoexponentiële aard in geval van een proefpersoon en van biexponentiële aard in de 5 anderen, waarbij in alle gevallen de terminale exponent in termen van statistische momenten zwaar domineerde. De tijdconstant $\tau_{term}$ van deze terminale exponent bedroeg gemiddeld (± SD) 6.7 ± 2.0 uur ($\tau_{\frac{1}{2}} = 4.7 ± 1.4$ uur). Een opvallend lange tijdconstant $\tau_{term} = 9.9$ uur werd waargenomen in een proefpersoon. Vanwege de dominantie van de terminale exponent benadert de gemiddelde verblijftijd (± SD) van CAF in het lichaam (6.5 ± 2.0 uur) de waarde van $\tau_{term}$, en is de variantie van de lichaamsverblijftijden (48 uur²) ongeveer het kwadraat van de gemiddelde verblijftijd.

Het gemiddelde ± SD van de plasmaklaring en het schijnbare distributievolume van CAF bedroeg in onze proefpersonen ongeveer $7.2 ± 3.6 l/uur$ en $42 ± 8 l$, respectievelijk. De grootte van het verdelingsvolume komt ongeveer overeen met het volume van het totale lichaamswater.

Per rondgang in de bloedbaan werd een fractie (de extractieratio) van 2.0 ± 1.0% van de CAF-moleculen geëlimineerd. De tijd voor een rondgang in de circulatie (transittijd) en het aantal rondgangen dat een CAF-molecuul kon maken alvorens te worden geëlimineerd bedroeg gemiddeld over moleculen en proefpersonen 7.0 ± 1.3 min, en 56 ± 17, respectievelijk.

Dezelfde vrijwilligers aan wie CAF per iv infusus was toegediend kregen bij twee andere gelegenheden koffie en thee te drinken, zoals beschreven in Hoofdstuk 12. CAF, toegediend in deze beiden dranken, werd zo goed als volledig uit de maag-darm tractus opgenomen (99 ± 4% voor CAF in koffie, 95 ± 6% voor CAF in thee). De opname geschiedde vrij snel de mediane opnametijd (waarop de helft van de aangeboden hoeveelheid is opgenomen) bedroeg voor CAF toegediend als koffie 20 ± 3 min, en voor CAF in thee 19 ± 3 min. De gemiddelde opnametijd lag iets hoger door 'slepen' van het absorptieproces 26 ± 5 min voor CAF in koffie, 23 ± 3 min voor die in thee. De verschillen tussen koffie en thee betreffende mate en snelheid van CAF-opname waren klein en nonsignificant. De profielen van de opnamesnelheid uitgezet tegen de tijd kenden een grillig verloop voor alle proefpersonen.
Het distributievolume van CAF bleef voor elk van de proefpersonen ongeveer gelijk gedurende de experimenten. De totale klaring (CL) en de tijdsconstante van eliminatie (\( \tau_{\text{term}} \)) vertoonden daarentegen aanzienlijke intra-individuele fluctuaties van experiment tot experiment. Geconcludeerd werd dat de verwijderskinetiek van CAF in onze proefpersonen naar alle waarschijnlijkheid dosisafhankelijk (non-lineair) was. CL en \( \tau_{\text{term}} \) waren in de theeproef, waarin verreweg de laagste CAF-dosis werd toegediend, significant hoger en lager, respectievelijk, dan in de koffieproef en na het \( \nu \) infuus van CAF.

Vergelijking van CL en \( \tau_{\text{term}} \) in beide laatsgenoemde experimenten leverde als conclusie dat de eliminatiesnelheid van CAF in onze proefpersonen tevens sterk afhankt van hun urineflow, die na toediening van koffie (en thee) significant hoger was dan na het infuus (mede door het eigen volume van de koffie/thee). Deze afhankelijkheid laat zich terugvoeren op de veronderstelde dosisafhankelijkheid van de verwijderskinetiek van CAF. Aangezien namelijk de renale klaring van CAF zelf zeer klein is, komt de koppeling tussen CAF-eliminatie en urineflow waarschijnlijk tot stand via de 3 dimethylxanthines, waarin CAF voor \( \pm 95\% \) wordt omgezet in de eerste fase van zijn metabolisme.

De dimethylxanthines plegen competitie met CAF om een of meer enzymen van cytochroom P-450, die de demethylering van xanthines bewerkstelligen, en worden tevens in aanzienlijke mate renaal geklaard. Verhoging van de urineflow bespoedigt de renale eliminatie van de dimethylxanthines, verlicht de competitie van CAF met deze stoffen om P-450, en leidt zo ook tot een snellere verwijdering van CAF.

In Hoofdstuk 13 wordt de kinetiek van CAF vergeleken met die van zijn voornaamste metabo routine paraxanthine (1,7-dimethyl xanthine, PX) in de 3 mannelijke vrijwilligers die eerder in de CAF-experimenten hadden geparticipeerd. De lichaamstransportfunctie \( \psi(t) \) was monoexponentieel in twee van hen en biexponentieel in de resterende persoon, waarbij net als bij CAF de terminale exponent domineerde. De farmacokinetische parameters van CAF en PX verschilden weinig. De tijdsconstante van eliminatie \( \tau_{\text{term}} \) bedroeg voor PX gemiddeld (± SD) 4,6 ± 0,9 uur (voor CAF 5,9 ± 1,9 uur). De plasmaklaring en het verdrijvingvolume van PX namen gemiddelde (± SD) waarden aan van respectievelijk 11,8 ± 2,2 l/uur en 52 ± 5 l (CAF 8,9 ± 4,8 l/uur, en 45 ± 9 l).

Na orale toediening in oplossing werd PX snel (gemiddelde opnametijd 22 ± 1 min) en nagenoeg volledig (94 ± 10 %) opgenomen (CAF 23 ± 7 min, en 103 ± 13 %, respectievelijk).
De gelijkenis van de tijdsconstanten van eliminatie van CAF en PX is er de oorzaak van dat de plasmaconcentratie curve van PX na toediening van een enkele dosis van de moederverbinding CAF een typerend vonvex profiel vertoont, dat zich vaak niet aan een som van exponentiële functies laat fitten, maar des te beter aan een zgn. 'gamma-functie'.

Van een intraveneuze dosis CAF werd 77 ± 7 % in PX omgezet door de 3 vrijwilligers, terwijl dit percentage voor een even grote orale dosis 84 ± 9 % bedroeg. Aangezien een 'first-pass' metabolisme voor CAF nog nimmer werd gerapporteerd in de literatuur, moet de oorzaak van het verschil waarschijnlijk gezocht worden in de urineflow van de proefpersonen. Deze was mede als gevolg van het grote volume water waarmee de orale dosis CAF werd toegediend, belangrijk hoger na orale dan na intraveneuze toediening van CAF. Waarschijnlijk werd door een relatief snelle (renale) verwijdering van de door de-methylering uit CAF gevormde dimethylxanthines (waaronder PX) dit metabole pad (de-methylering van CAF) na orale toediening van CAF meer benadrukt ten koste van andere metabole routes, dan na i.v. toediening van CAF het geval was.

In Hoofdstuk 14 tenslotte komt de kinetiek van kinine aan bod, zowel na een intraveneus infuus als na orale toediening van een gelatine capsule met het sulfaatzout van de stof aan 7 jonge gezonde proefpersonen (4 mannen, 3 vrouwen). De lichaamstransportfunctie \( \psi(t) \) was biëxponentieel in alle vrijwilligers. De tijdsconstanten van de 2 exponenten lagen ver uiteen en bedroegen gemiddeld (± SD) 3.6 ± 0.7 min, en 12.3 ± 1.8 uur. In termen van statistische momenten was de terminale fase veruit dominant. De gemiddelde verblijftijd van kinine in het lichaam (12.0 ± 1.8 uur) was dan ook bij goede benadering gelijk aan \( t_{\text{term}} \). De totale kinineklaring in de proefpersonen bedroeg 8.1 ± 1.4 l/uur, het distributievolume 96 ± 18 l.

Gemiddeld over proefpersonen en moleculen deden de kininemoleculen 16 ± 3 min over een circulatoire transit door het lichaam, en completeerden zij 45 ± 8 transits alvorens te worden verwijderd uit de bloedcirculatie. Per transit werd een fractie (de extractie ratio) van 2.3 ± 0.4 % van de kininemoleculen geëlimineerd, waarvan een minderheid (ongeveer 1/6 deel) door uitscheiding in onveranderde vorm in de urine. De renale klaring van kinine bedroeg ongeveer 1.3 ± 0.2 l/uur. Deze waarde duidt op tubulaire secretie van kinine, aangezien de aan plasma-eiwitten gebonden fractie van kinine ongeveer 90 % bedraagt.

Na orale toediening van kininesulfaat per gelatine capsule werd kinine in hoge mate (90 ± 18 %) in de bloedbaan geabsorbeerd. Het absorptieprofiel van de stof was regelmatig en symmetrisch in de vrijwilligers, hetgeen kan worden
opgemaakt uit het bijna samenvallen van mediane, modale, en gemiddelde absorptietijd (respectievelijk 1,25 ± 0,38 uur, 1,26 ± 0,41 uur, en 1,42 ± 0,35 uur). De traagste absorptie werd waargenomen in een vrijwilliger, van wie later werd vastgesteld dat zij leed aan coeliacie.

In twee vrijwilligers deed zich geruime tijd na de eerste absorptiepiek nog een tweede absorptiefase voor, waarbij nog eens 11% en 6% van de toegediende hoeveelheid werd opgenomen. Daar deze tweede fase zich vlak na de lunch voordeed, is de inname van voedsel een mogelijke verklaring voor het hernieuwde optreden van absorptie. De vondst van kleine hoeveelheden glucuronide-conjugaat van kinine in de urine van uitsluitend deze zelfde twee vrijwilligers zou ook kunnen wijzen op een hepato-enterale kringloop van kinine als verklaring voor de dubbele absorptiepiek, waarbij wederom de lunch het 'startschot' zou kunnen zijn dat de kringloop in gang zet. Daar in de urine van genoemde twee proefpersonen wel na orale, maar niet na intraveneuze toediening kinine-glucuronide werd aangetroffen, is het mogelijk dat glucuronideering van kinine gerelateerd is aan de 'first pass'. Op grond van de beschikbare gegevens was het niet mogelijk een nadere uitspraak over de oorzaak van het fenomeen van de dubbele absorptiepieeken te doen.
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In januari 1985 trad de auteur van dit proefschrift in dienst van de Katholieke Universiteit Nijmegen. Tot medio december 1987 was hij werkzaam als wetenschappelijk medewerker op het Farmacologisch Instituut. Als lid van de onderzoeksgroep Farmacologische Systeemdynamica verrichtte hij onder leiding van Prof Dr J M Van Rossum onderzoek naar de klinische farmacokinetiek van de plantaardige genotmiddelen nicotine, cafféïne en kinine. De interpretatie van de onderzoeksresultaten geschiedde in het raamwerk van een moderne model-onafhankelijke systeemdynamische benadering van de farmacokinetiek. De onderzoeksresultaten zijn beschreven in deze dissertatie.

Buiten het kader van het proefschrift werkte schrijver dezes aan een systeemdynamische benadering van de kinetiek van de hepato-enterale kringloop. Empirische onderbouwing van de theorie werd verkregen door dierexperimenten aan de modelstof phenolphthaleïne en het glucuronide-conjugaat van deze stof. Daarnaast nam hij het voortouw tot de synthese van de laag-calorische plantaardige zoetstof hernandulcine, en de bestudering van de kinetiek en de fysisch-chemische eigenschappen van deze stof.

Abstracts of Oral Communications and Presentations by Poster


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Teeuwen HWA De absolute inname van nicotine en koolmonoxide door rokers van conventionele en geventileerde filtersigaretten Een farmakokinetische aanpak Voordracht op de landelijke meeting 'Roken / Niet Roken', georganiseerd door de Stichting Volksgezondheid en Roken, 7 oktober 1987, Utrecht
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- Van Rossum JM, Teeuwen HWA, Van Lingen G, Maes RAA Farmacokinetiek studies van het verblijf van geneesmiddelen en giften in het lichaam volgens een klassieke descriptieve methode en een moderne operationele systeemdy-namische visie Forum Diagnosticum (Boehringer Ingelheim Corporate Magazine) 3/1986 pp 59-64

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- Teeuwen HWA, Bosman R, Hissink M, Aalders RJW, Van Rossum JM Nicotine intake by smokers of filter cigarettes Part I conventional filter cigarettes (ch 8 of this thesis) In preparation
- Teeuwen HWA, Bosman R, Hissink M, Aalders RJW, Van Rossum JM Nicotine intake by smokers of filter cigarettes Part II ventilated filter cigarettes (ch 9 of this thesis) In preparation
- Teeuwen HWA, Aalders RJW, Van Rossum JM Carbon monoxide intake by smokers of conventional and ventilated filter cigarettes (ch 10 this thesis) In preparation
- Teeuwen HWA, De Schepper PJ, Tjandramaga TB, Van Rossum JM Intravenous single dose kinetics of caffeine in man a dynamic systems approach (ch 11 of this thesis) In preparation
- Teeuwen HWA, De Schepper PJ, Tjandramaga TB, Van Rossum JM Bioavailability of caffeine in coffee and tea, and dependence of caffeine total clearance on dose and urine flow (ch 12 of this thesis) In preparation
- Teeuwen HWA, De Schepper PJ, Tjandramaga TB, Van Rossum JM Clinical pharmacokinetics paraxanthine, the main caffeine metabolite in man (ch 13 of this thesis) In preparation
- Teeuwen HWA, De Schepper PJ, Tjandramaga TB, Van Rossum JM Oral and intravenous single dose kinetics of quinine in healthy man (ch 14 of this thesis) In preparation
• Teeuwen HWA, De Bie JFM, Van Rossum JM Pharmacokinetics of the enterohepatic cycle of phenolphthaleine in the Beagle A dynamic systems approach In preparation

Bioassays

• Teeuwen HWA, Aalders RJW, Van Rossum JM, Maes RAA Simultaneous estimation of nicotine and cotinine levels in biological fluids using high resolution capillary-column gas chromatography combined with solid phase extraction work-up (ch 2 of this thesis) In preparation
• Teeuwen HWA, Elbers EL, Van Rossum JM, Maes RAA Rapid and sensitive gas chromatographic determination of caffeine in beverages and biological fluids (ch 5 of this thesis) Submitted for publication
• Teeuwen HWA, Van Rossum JM, Maes RAA Selective high-performance liquid chromatographic method for estimating quinine levels in biological fluids (ch 7 of this thesis) Submitted for publication
STELLINGEN

behorend bij de dissertatie

"CLINICAL PHARMACOKINETICS OF NICOTINE, CAFFEINE, AND QUININE"

"A Systems Dynamics Approach"

HARALD W A. TEEUWEN
NIJMEGEN/BRUSSEL, 23 DECEMBER 1987
De in de farmacokinetische compartimentenleer gangbare wiskundige benadering van het fenomeen absorptie als een (meestal na een dode tijd) momentaan aanzwellend, en daarna strikt monoeexponentieel aflopend gebeuren is fysiologisch laakbaar en dient te worden verworpen ten gunste van de berekening van de absorptiesnelheidscurve als functie van de tijd.

**Deze dissertatie**

De vraag gesteld in de titel van het artikel van Ashton et al. "Should intake of carbon monoxide be used as a guide of intake of other smoke constituents?" lijkt voor rookbestanddelen die grotendeels of geheel in de aerosolfase van de rook voorkomen, zoals nicotine, waarschijnlijk met "neen" te moeten worden beantwoord.


**Deze dissertatie**

De gevolgtrekking van Bonati et al. als zou de kinetiek van caffeine niet dosis-afhankelijk zijn bij doses en plasmaspiegels van het alkaloid waaraan de doorsnee gebruiker van cafeïnehoudende dranken zich blootstelt is gebaseerd op een zeer beperkt aantal waarnemingen, en wordt in twijfel getrokken door getableerde gegevens in het artikel zelf.


**Deze dissertatie**

De wijze waarop Berlin et al. het schijnbare verdelingsvolume van kinine in het menselijk lichaam berekenen is discutabel.

CM Berlin, JM Stackman, ES Vesell, Clin Pharm Ther 18 (1975) 670

**Deze dissertatie**

Een eenvoudig inhaleertoestel dat een stootgewijze afgifte en absorptie van nicotine in de alveoli kan bewerkstelligen, zou als hulpmiddel om te stoppen met roken wellicht meer effect sorteren dan de weinig succesvolle nicotinekauwgom.

Russel MAH, and Feyerabend C, Cigarette smoking: a dependence on high-nicotine boli, Drug Metab Rev 8 (1978) 29

**Deze dissertatie**

De zin van de wettelijk verplichte nicotine- en teergetallen op sigarettenpakjes zou moeten zijn, een realistische voorspelling te leveren van de inname van nicotine en teer door de doorsnee menselijke roker van sigaretten van het merk in kwestie. Als genoegzaam duidelijk is dat menselijke rook patronen tussen sigarettenklassen wezenlijk verschillen, zou dienovereenkomstig de standaard machinale rookprocedure, waarmee de nicotine en teergetallen worden bepaald, naar sigaretten-klasse moeten worden gedifferentieerd.

**Deze dissertatie**

"Actieve" rokers dienen op basis van goede omgangsvormen de irritatie en het lichamelijk ongemak, die/door het ongewild inhaleren van met tabaksrook verontreinigde lucht voor velen in deze samenleving met zich meebringt, zoveel mogelijk te voorkomen, onafhankelijk van de mate waarin de gezondheid door "passief" roken wordt bedreigd.

**Deze dissertatie**

De door de consument zo gewaardeerde "natuurlijke" (biogene) herkomst van levens- en genotmiddelen en cosmetica en van eventueel daarin verwerkte
kleur-, geur-, en smaakstoffen garandeert geenszins de onschadelijkheid voor de gezondheid van dergelijke middelen en additieven.

IX
Het poneren van stellingen bij een dissertatie biedt de promovendus een goede mogelijkheid tot het ad dissertationem ventileren van eventuele beleidsmatige of politiek getinte consequenties van zijn werk, die in het wetenschappelijke kader van de dissertatie zelf minder op hun plaats zijn. Mede daarom moet de afschaffing van de stellingen aan de Rijks Universiteit Utrecht per 1 september 1987 jongstleden dan ook worden betreurd.

X
Na de invoering van het facultair "ongedifferentieerde" doctoraat aan de K.U. Nijmegen per 1 januari 1985 lijkt de titelpagina van Nijmeegse dissertaties een onhandig geformuleerd en inhoudelijk gekunsteld compromis tussen vóór- en tegenstanders van het noemen van de facultaire indeling. De zinsnede "Een wetenschappelijke proeve op het gebied van ... (volgt: faculteitsnaam zonder het woord "faculteit")" is weinig informatief, doet afbreuk aan de volwaardigheid van het in de dissertatie beschreven werk, en kan beter in zijn geheel worden weggelaten.

XI

Nijmeegs Universiteits Blad 5 okt. 1962, K.U. Nieuws 31 okt. 1985

XII
De authoriteit die uitgaat van de opdruk op sigarettepakjes, luidend: "Roken Bedreigt de Gezondheid. De Minister van Volksgezondheid en Milieuhygiëne" wordt ondergraven door het niet bestaan van genoemde minister.

XIII
De in de lekenpers veelvuldig gehanteerde woordcombinatie "chemische stoffen" is een schoolvoorbeeld van een pleonasme.

XIV
Het sociaal gedrag van medewerkers van een farmacokinetisch laboratorium kan onderhevig zijn aan beïnvloeding door de vluchtigheidskarakteristieken van de in het laboratorium onderzochte genees- of genotmiddelen.

Deze dissertatie.

XV
Te oordelen naar het verbijsterend lage salaris dat wordt toegekend aan assistenten in opleiding (A.I.O.'s) in met name het beginjaar van hun "tweede fase" zou de afkorting van hun functie evenals hun portemonnaie zonder gevaar voor verlies van inhoud omgedraaid kunnen worden tot "O.I.A.": "Onderzoeker In Armoede "

XVI
This unimpeachable thesis contains five a's, two b's, three c's, two d's, twenty-six e's, six f's, two g's, eight h's, twelve i's, four l's, two m's, fourteen n's, eleven o's, two p's, seven r's, thirty-one s's, twenty t's, five u's, seven v's, nine w's, three x's, and four y's.