pharmacokinetics of diuretic drugs

studies with chlorthalidone and mefruside in man

harry l.j.m. fleuren
PHARMACOKINETICS OF DIURETIC DRUGS

STUDIES WITH CHLORTHALIDONE AND MEFURUSIDE IN MAN
PROMOTORES:
PROF. DR. J. M. VAN ROSSUM
EN
PROF. DR. C. A. M. VAN GINNEKEN
PHARMACOKINETICS OF DIURETIC DRUGS

STUDIES WITH CHLORTHALIDONE AND MEFRUSIDE IN MAN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. P.G.A.B. WIJDEVELD VOLGENS BESLUIT VAN HET COLLEGE VAN DE CANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 6 SEPTEMBER 1979 DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

HENDRIK LAMBERT JOZEF MARIA FLEUREN

GEBOREN TE NIJMEGEN

1979
GRAFISCH BEDRIJF H.W. JANSSEN B.V., GENNENP THE NETHERLANDS
aan mijn ouders
voor Marion, Laurens en Emmeke
CONTENTS

SECTION I
GENERAL INTRODUCTION

CHAPTER 1 DIURETIC AGENTS
AND PHARMACOKINETICS

SULFONAMIDE DIURETICS 21
RENAL SITES OF ACTION OF SULFONAMIDE DIURETICS 23
RENAL TRANSPORT OF SULFONAMIDE DIURETICS 26
Relationship between the effect of diuretic drugs and their own urinary excretion 26
PHARMACOKINETICS: GENERAL REMARKS 31
HUMAN PHARMACOKINETICS
OF SULFONAMIDE DIURETICS 36
Volumes of distribution 36
Uptake in red blood cells 36
Plasma protein binding 38
Glomerular filtration, tubular secretion and reabsorption 38
Plasma clearance 39
Pharmacokinetics and diuretic effects 40
THE INVESTIGATIONS DESCRIBED IN THIS THESIS 42
REFERENCES 43
CHAPTER 4  DETERMINATION OF MEFRUSIDE IN PLASMA, RED BLOOD CELLS AND URINE

INTRODUCTION 92
MATERIALS 93
Reagents 93
Gas chromatography 94
Other apparatus 94
METHODS 95
Sampling of blood and urine 95
Selection of derivatization time and temperature 95
Analytical procedure 96
DRUG PARTITIONING BETWEEN PLASMA AND RED BLOOD CELLS 96
Rate of distribution in whole blood at 20 and 37°C 96
Influence of temperature upon the distribution equilibrium 97
RESULTS AND DISCUSSION 98
Gas chromatography and specificity 98
CHAPTER 5 SEPARATE ASSAY OF MEFRUSIDE METABOLITES IN THE LACTONE AND OPEN ACID CONFORMATION

INTRODUCTION 110
MATERIALS AND METHODS 111
Drugs 111
Derivatization and gas chromatography 112
pH-dependent distribution between aqueous and organic phases 113
Procedure for the extraction of 5-oxo-mefruside and its hydroxy acid analogue from biological samples 114
Sampling of blood and urine, in vitro distribution between plasma and red cells 115
Comparison of presumed metabolite with synthetic 5-oxo-mefruside by gas chromatography - mass spectrometry 115
RESULTS AND DISCUSSION 116
Drug partitioning 116
Gas chromatographic determination of 5-oxo-mefruside and its open acid counterpart in plasma, red blood cells and urine 117
Identification of mefruside metabolites in biological samples 117
In vitro distribution of mefruside metabolites between plasma and erythrocytes 121
SUMMARY 122
REFERENCES 123
CHAPTER 6  DIFFERENCE POTENTIOMETRIC METHOD FOR DETERMINING DISSOCIATION CONSTANTS OF VERY SLIGHTLY WATER-SOLUBLE DRUGS, APPLIED TO THE SULFONAMIDE DIURETIC CHLORTHALIDONE

INTRODUCTION 124
EXPERIMENTAL 125
RESULTS AND DISCUSSION 126
SUMMARY 131
REFERENCES 131

SECTION III

PHARMACOKINETICS OF CHLORTHALIDONE

CHAPTER 7  BASIC PHARMACOKINETIC CHARACTERISTICS OF CHLORTHALIDONE

INTRODUCTION 135
MATERIALS AND METHODS 136
RESULTS 137
Time course of chlorthalidone concentration in plasma and red blood cells 137
Urinary excretion 138
Distribution of chlorthalidone between plasma and red blood cells 139
PHARMACOKINETIC ANALYSIS 141
Linear approach 141
Non-linear model 141
DISCUSSION 147
CONCLUSION 150
SUMMARY 151
APPENDIX 7.1 151
APPENDIX 7.2 153
REFERENCES 155
CHAPTER 10  URINARY EXCRETION OF CHLORTHALIDONE AS A FUNCTION OF DOSE

INTRODUCTION  201
METHODS  201
Subjects and dosage  201
Biological samples and assay  202
Pharmacokinetic analysis  202
RESULTS  203
Urinary excretion rate vs time, comparison with plasma and red blood cell concentration decay  203
Cumulative urinary excretion  205
Renal clearance  205
Dependence of renal clearance upon urinary flow and pH  209
DISCUSSION  211
Dose-dependent urinary excretion  211
Fluctuations in renal plasma clearance  212
Therapeutic meaning  213
SUMMARY  213
REFERENCES  214

CHAPTER 11  BILIARY EXCRETION OF CHLORTHALIDONE IN HUMANS

INTRODUCTION  216
EXPERIMENTAL PROCEDURE  216
Patients  216
Chlorthalidone assay  218
SECTION IV

PHARMACOKINETICS OF MEFRUSIDE

CHAPTER 14 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION OF MEFRUSIDE IN MAN

INTRODUCTION 251
MATERIALS AND METHODS 252
Drug administration and sampling of blood and urine 252
Assay of mefruside and metabolites 253
Pharmacokinetic analysis 253
RESULTS 254
Pharmacokinetics of mefruside in plasma 254
Uptake of mefruside in red blood cells 257
Urinary excretion of mefruside 258
Concentration of mefruside metabolites in plasma and red blood cells 258
Urinary excretion of 5-oxo-mefruside and its hydroxy-carboxylic acid analogue 260
Urinary excretion of conjugated metabolite 263
DISCUSSION AND CONCLUSIONS 263
Pharmacokinetic parameters 263
Binding to red blood cells 264
Intersubject difference in elimination of mefruside 265
Relationship between pharmacokinetics and diuretic effect 265
SUMMARY 266
REFERENCES 267

CHAPTER 15 DIURETIC EFFECTS OF MEFRUSIDE AND ITS MAIN METABOLITES IN THE ISOLATED PERFUSED RAT KIDNEY

INTRODUCTION 269
MATERIALS AND METHODS 270
Drugs 270
The isolated perfused rat kidney 271
DIURETIC AGENTS AND PHARMACOKINETICS

SULFONAMIDE DIURETICS

In principle, diuretics might be called all agents which promote the excretion of salt and water in the urine. Mercurous chloride was applied as a diuretic already by Paracelsus and its use remained quite popular until nearly 1900 (Heidenreich, 1969). This compound, however, and the organomercurial diuretics which originate from it have now become obsolete. Also the xanthine derivatives, e.g. aminophyllin, which were introduced about 100 years ago (Füllgraff, 1969) have largely been abandoned and serve only as adjunctive agents in certain resistant cases of oedema.

This study will be confined to the diuretics of the sulfonamide-type. Together with a few compounds of different chemical nature, e.g. ethacrynic acid, triamterene, amiloride or spironolactone, these drugs constitute the mainstay of diuretic therapy nowadays. They have the advantage of low toxicity and oral effectiveness over the organomercurials, and can exert a greater natriuretic effect than the xanthine diuretics. The history of the sulfonamide diuretics begins with a side-effect of antibacterial therapy. After the observation that sulfanilamide was capable of producing a metabolic acidosis together with alkalinization of the urine and an increase of sodium bicarbonate excretion (Southworth, 1937), it was shown that renal carbonic anhydrase was inhibited, suggesting that this enzyme was involved in the tubular reabsorption of bicarbonate (Mann and Keilin, 1940). The inhibition of carbonic anhydrase derived from red blood cells was used subsequently as an in vitro test system to develop more effective diuretic agents. This led to the synthesis of acetazolamide (Roblin and Clapp, 1950), upon which many pharmacological studies have been centered (Maren, 1967).

The efforts of Beyer and Baer (1961), however, concentrated on the search for drugs which promoted the excretion of chloride rather than that of bicarbonate, because they realized that there was no consistent correlation between in vitro carbonic anhydrase inhibition and in vivo natriuretic potency. Molecular modification of sulfonamide compounds resulted eventually in the introduction of chlorothiazide, hydrochlorothiazide and
many other benzothiadiazines with an unsubstituted sulfonamide group (Fig. 1.1). The structure-activity relationships within this group have been reviewed by Peters and Roch-Ramel (1969a).

![Diagram of sulfonamide diuretics]

**Figure 1.1**

The individual thiazides differ largely in natriuretic potency (i.e. the dose needed to produce a similar effect), but their log dose-response curves are parallel (Fig. 1.2) and the maximum diuretic effect that can be achieved is within the experimental error the same (Peters and Roch-Ramel 1969a; Meng and Loew, 1974). The same holds true for the so-called heterocyclic variants of the benzothiadiazines. These variants include phthalimidines (chlorthalidone (Fig. 1.1); clorexolone), quinazolinones (quinethazone; metolazone), benzenedisulfonamides (mefruside, Fig. 1.1) and chlorobenzamides (clopamide) (Davies and Wilson, 1975; Wilson and Kirkendall, 1970; Suki et al., 1965).

In contrast with the above group of compounds, furosemide and...
bumetanide (Fig. 1.1) can induce much larger losses of sodium chloride. Expressed as a fraction of the total sodium load filtered through the glomeruli, as much as 15-25% can be excreted in the urine, compared with a 5-8% loss of sodium chloride after maximum doses of the thiazides and their heterocyclic variants (Davies and Wilson, 1975; Goldberg, 1973). To understand the differences in the pattern and extent of action between the primary carbonic anhydrase inhibitors, like acetazolamide, the moderately potent thiazides, including chlorthalidone and mefruside, and the very potent drugs furosemide and bumetanide, a brief description of the normal physiological events in the kidney should be of value and will be presented in the next section.

Figure 1.2

RENAL SITES OF ACTION OF SULFONAMIDE DIURETICS

A standard human subject forms about 170 liters of glomerular filtrate each day, of which only 1-2 liters are excreted as urine. The reabsorption of filtered solutes and water is clearly the main physiological task of the kidney, and this demands the largest part of its available metabolic energy (Maude, 1974). During the past three decades, numerous efforts have been made to unravel the specific mechanisms of reabsorption within the nephron and the sites where diuretics act. The resulting insights have been summarized in several reviews (Goldberg, 1973; Suki et al., 1973; Davies and Wilson, 1975; Jacobson and Kokko, 1976): under normal circumstances,
50-60% of salt and water reabsorption occurs isosmotically in the proximal tubules, i.e. the fluid at the end of the tubule has the same osmolality as plasma (Fig. 1.3). In the descending limb of the loop of Henle, water is lost to the hypertonic medullary interstitium and finally the fluid is hypertonic at the tip of the loop. At or near the bend of the loop the tubular cells begin to reabsorb sodium chloride: this process continues throughout the entire length of the thick ascending limb of Henle and removes about 25% of the filtered load. Although classical experiments had indicated that active sodium transport was the driving force behind this net transport of sodium chloride (see Goldberg, 1973; Fig. 1.3), recent studies provided evidence rather that active chloride transport is involved in this part of the nephron (Jacobson and Kokko, 1976). Irrespective of this controversy on the mechanism, dilute urine is generated at the end, because the ascending limb of the loop of Henle is practically impermeable to water. In the distal convoluted tubule another 5% of the filtered sodium load is reabsorbed in exchange for hydrogen and potassium ions, mainly under control of aldosterone. The fluid leaving the distal tubule is hypotonic, but can become isotonic and even hypertonic in the collecting duct under influence of the antidiuretic hormone. This hormone regulates the water-permeability of the collecting ducts; under conditions of hydropenia, fluid is reabsorbed into the hypertonic medulla, so that a concentrated final urine results (see Fig. 1.3). Under conditions of a large water intake, this segment of the nephron is impermeable to water and a dilute urine is excreted.

The sites of action of diuretic drugs within the nephron have been localized mainly by means of osmolar clearance studies and micropuncture techniques. An excellent review of the reasoning which led to a definite assessment of these sites has been given by Goldberg (1973). This information is summarized in Table 1.1. Carbonic anhydrase inhibitors diminish hydrogen ion secretion and bicarbonate reabsorption in both the proximal tubule and in the distal nephron. The thiazides, including chlorthalidone and mefruside, are not entirely devoid of carbonic anhydrase inhibition, but exert their major natriuretic and chloruretic effects in the cortical portion of the ascending limb of the loop of Henle. The most potent diuretic agents - furosemide, bumetanide, the new drug piretanide (Teredesai and Puschett, 1979) and also ethacrynic acid and the afore-mentioned organomercurials - inhibit sodium chloride reabsorption over the entire length of the ascending limb of the loop of Henle. Due to their action in the medullary segment the medulla is made less hypertonic than normal, so that also the driving force for water reabsorption in the collecting duct is drastically reduced.

Compared to the information on the sites of action of diuretic drugs, still little is known about the molecular modes of action of these agents. The
Figure 1.3
Diagram of sites of sodium and water reabsorption in the nephron. Solid arrows denote active transport, broken arrows passive transport, and the angulated arrow indicates water impermeability. Site 1: paired sodium chloride reabsorption in proximal tubule; site 2: proximal hydrogen ion secretion and sodium bicarbonate reabsorption; site 3: sodium chloride reabsorption (without water) in the medullary portion of ascending limb of Henle’s loop; site 4: sodium chloride reabsorption (without water) in the cortical portion of the ascending limb; site 5: distal hydrogen and potassium secretion in exchange for sodium, regulated by aldosterone; site 6: aldosterone-independent distal cation secretion and sodium reabsorption. The water-permeability of the collecting duct is regulated by the antidiuretic hormone. Reproduced, with permission, from Goldberg (1973).

Table 1.1

<table>
<thead>
<tr>
<th>Diuretic drug</th>
<th>Proximal tubule</th>
<th>Ascending limb of loop of Henle</th>
<th>Distal tubule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>medullary segment</td>
<td>cortical segment</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>+ (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td>( + ) (2)</td>
<td>+ (4)</td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td>( + ) (1,2)</td>
<td>+ (3)</td>
<td>( + ) (3)</td>
</tr>
</tbody>
</table>

a A representative compound of each class has been taken; 
b Numbers in parentheses refer to functional sites indicated in Fig 1.3; +, major site of action; ( + ) minor site of action; c = possible distal inhibitory effect on hydrogen secretion via inhibition of carbonic anhydrase. Data obtained from references quoted in the text.
early concept of direct inhibition of renal carbonic anhydrase as the primary working mechanism has almost completely been set aside for other sulfonamide diuretics than acetazolamide and congeners because of: 1) the poor correlation of in vitro carbonic anhydrase inhibitory activity with in vivo natriuretic potency within the thiazide group, 2) the mainly chloruretic properties of the thiazides in contrast with the elevated bicarbonate excretion observed after administration of acetazolamide (Meng and Loew, 1974; Peters and Roch-Ramel, 1969a). Several other mechanisms have been proposed. Interferences with \( \text{Na}^+, \text{K}^+ \)-ATPase, with glycolysis, oxidative metabolism, plasma membrane adenylate cyclase, and enzymatic prostaglandin degradation, have been demonstrated to be involved under various in vitro conditions, but no common mechanism has emerged (Jacobson and Kokko, 1976; Meng and Loew, 1974; Peters and Roch-Ramel, 1969a, Benet, 1979).

RENAL TRANSPORT OF SULFONAMIDE DIURETICS

Sulfonamide diuretics concentrate in renal tissue to a manyfold of their plasma concentration (Peters and Roch-Ramel, 1969a; Pulver et al., 1959; Beisenherz et al., 1966; Duhm et al., 1967; Taugner and Iravani, 1965; Cohen et al., 1976). In vitro incubations of the drugs with kidney slices showed that only the cortical part and not the medullary tissue is responsible for this uptake (Beyer and Baer, 1975; Peters and Roch-Ramel, 1969a; Weiner, 1973). It is generally accepted that the organic anion transport system of the kidney, by which well-known substrates as para-aminohippuric acid, phenol red, penicillin, probenecid and many other acidic compounds are secreted, is used also for the elimination of acetazolamide, the thiazides and the potent diuretics furosemide and bumetanide (Beyer and Baer, 1961, 1975; Despopoulos, 1965; Deetjen, 1966; Hook and Williamson, 1965; Olsen, 1977; Peters and Roch-Ramel, 1969a; Weiner, 1973). The site of accumulation and secretion into the lumen has been localized exclusively in the proximal part of the nephron, especially the pars recta (Beyer and Baer, 1961; Bowman, 1975; Deetjen, 1966; Taugner and Iravani, 1965; Weiner, 1973).

Relationship between the effect of diuretic drugs and their own urinary excretion.

In the first years after the introduction of the benzothiadiazines, this site of accumulation - the proximal tubular cells - was supposed to be identical with the site of natriuretic action of these drugs. This hypothesis seemed to be strengthened by a good correlation between the rate and extent of prox-
imal uptake and natriuretic potency in a series of thiazides of varying lipophilicity (Beyer and Baer, 1961). However, as discussed in the previous section, there is overwhelming evidence now that the major sites of action of both the thiazides and the loop diuretics are not localized in the proximal, but in the distal part of the nephron. Furthermore, studies with isolated kidney tubules have demonstrated that rather the intratubular concentration than that at the peritubular membrane is decisive for the inhibition of sodium chloride reabsorption by furosemide in the thick ascending limb of Henle (Deetjen, 1966; Burg et al. 1973; Bowman, 1975). Also bumetanide and ethacrynic acid have been demonstrated to exert their diuretic effects from the luminal side of this nephron segment (Burg and Green, 1973; Imai, 1977). So, it appears that the proximal tubular secretion of these diuretics is merely a prerequisite for the diuretic effect that follows in a later part of the nephron. It can be visualized then that the urinary excretion rate of these drugs, which is a function of tubular secretion, reflects the amount of drug in the tubule lumen and thus might be a measure for the natriuretic response. This view is supported by the finding that changes

![Graph](image)

**Figure 1.4**
Following administration of a single dose of chlorthalidone to a healthy human subject, the excretion rates of urinary sodium and the diuretic itself change in almost identical direction.
in the urinary excretion rate of furosemide or bumetanide run closely parallel with changes in the urinary excretion of sodium. On the contrary, very poor correlations have been found between the plasma concentration and the diuretic effects of these drugs (Andreasen et al., 1978; Davies et al., 1974; Feit et al., 1973; Rose et al., 1976; Sörgel et al., 1979). The lack of a direct relationship between plasma concentration and natriuresis is indicated further by the way probenecid interacts with the diuretic response to furosemide and bumetanide administered as single doses. Probenecid competes for tubular secretion, so that the urinary excretion rate of both diuretics is reduced and the plasma concentration increases. Under these conditions a clear reduction of the diuretic effect has been reported (Hook and Williamson, 1965; Homeida et al., 1977; Honari et al., 1977; Lant, 1975).

Although direct proof for the dependence of natriuresis upon luminal concentration is still lacking for the diuretics of the thiazide class, there is a good agreement between the changes in urinary excretion rate of chlorthalidone and urinary sodium in man, see Fig. 1.4 (Fleuren and Boomkens, 1979). Furthermore, a reduction of the diuretic effects of chlorothiazide

![Figure 1.5](image)

Typical patterns of salt excretion during 5 days after single doses of chlorthalidone in man.
and hydrochlorothiazide in dogs after previous treatment with probenecid has been described (Beyer and Baer, 1961, 1975).

For a proper understanding of the interaction of probenecid with the natriuresis caused by the long-acting drug chlorthalidone, some information on the time course of action of the latter has to be given. A typical pattern of salt excretion after therapeutic doses of this drug in man is shown in Fig. 1.5. Three different single doses were administered on different occasions to the same healthy human subject, who had a controlled dietary intake of 10 gram sodium chloride (172 mEq Na) each day (during the trial and a preceding control period of 5 days). There was a dose-dependent increase of sodium excretion lasting for up to two days. Hereafter, net excretion became negative: such a "rebound-phase" is generally observed with diuretics if the losses of salt are not replaced during the experiment (Meng and Loew, 1974; Peters and Roch-Ramel, 1969a). This retention is due to a homeostatic maintenance of the sodium balance, which is controlled by hormonal factors (Laragh and Sealy, 1973). It can be discerned therefore from Fig. 1.5 that the extent of sodium retention in the rebound-phase is proportional to the magnitude of the preceding natriuretic effect.

Fig. 1.6 compares the time course of the plasma concentration and the urinary excretion rate of chlorthalidone with the pattern of urinary sodium excretion after single 100 mg doses of chlorthalidone to a human subject, without and with simultaneous administration of probenecid (Fleuren and Boomkens, 1979). There was a strong depression of the renal plasma clearance of chlorthalidone during the two days of a high probenecid plasma concentration and on the following day*. The average renal clearance in this period was only 27.5 ± 4.6 ml/min (mean ± S.D., n = 9), compared to 52.2 ± 13.4 ml/min (mean ± S.D., n = 17) in the experiment without probenecid. The resulting smaller amount of chlorthalidone excreted into urine was accompanied by a drastic reduction of the urinary sodium excretion. Correspondingly, also the rebound-retention of sodium on following days was much less marked than that seen without probenecid (Fig. 1.6, cf. Fig. 1.5).

Whether and to what extent such situation applies to all thiazides remains to be confirmed. Despite the fact that a considerable number of publications has appeared recently on the single dose kinetics of hydrochlorothiazide, no close correlation between urinary excretion rate and sodium losses has apparently been recognized (Beermann and Groschinsky-Grind, 1977, 1978; Beermann et al., 1976, 1977b). It is obvious, however, that such a correlation could easily be overlooked if the urine portions are not separately collected and assayed, but pooled instead.

* Probenecid concentration analysed by high-pressure liquid chromatography according to Hekman et al., 1979.
Figure 1.6
Effect of probenecid on the pharmacokinetics and the natriuretic effect of chlorthalidone in a healthy human subject. The two experiments were performed 1 month apart, each with 100 mg of the diuretic. The broken lines in the lower graphs indicate the average salt excretion during previous control periods.
For many drugs the plasma concentration is considered as an appropriate measure of drug response and several clear-cut relationships between plasma levels and biological effects have been established (Sjöqvist et al., 1976). On the contrary, however, as outlined above, it appears that for the sulfonamide diuretics investigated the amount of drug excreted with the urine instead of the plasma concentration is well correlated with the diuretic effect. This conclusion is not invalidated by the observation by Brater (1978), who reported a paradoxical increase in the 8-hours diuretic effect of a single dose of chlorothiazide in human subjects after pretreatment with probenecid. The dose administered, 0.5-1 gram intravenously, was fairly high so that the supra-maximal amounts of drug which would otherwise rapidly be eliminated were longer available now, because probenecid retarded their tubular secretion. Larger doses of sulfonamide diuretics apparently deliver sufficient amounts of drug at their site of action, so that the natriuresis is not reduced by probenecid, despite a lesser drug clearance. This has been confirmed for hydrochlorothiazide (Beyer and Baer, 1961, 1975). Also the well-known observation that probenecid does not interfere with the long-term treatment of hypertension by diuretics (e.g. Freis and Sappington, 1966; Garcia and Yendt, 1970) is in agreement with this concept.

PHARMACOKINETICS: GENERAL REMARKS

Several barriers must be passed by a drug before it can exert a biological effect. Three main phases can be discerned in this process as shown in the following scheme (cf. Ariëns and Simonis, 1974):

In the pharmaceutical phase the active principle has to be released from its dosage form, e.g. tablet, capsule or potion. The rate and extent of liberation are influenced by crystal structure, particle size and coating of the drug formulation.

The pharmacokinetic phase includes the absorption, distribution, biotransformation and excretion of drugs. These events proceed generally with a small rate compared with the very fast formation of drug-receptor
complexes in the later pharmacodynamic phase (van Ginneken, 1977). Therefore, the onset and time course of action of a drug will in many cases depend on the rate-limiting kinetic characteristics of that substance.

The use of open compartment models in pharmacokinetic studies is widely accepted and many textbooks and other readings on this matter are available (Teorell, 1937; Dost, 1953, 1968; Rescigno and Segre, 1966; Gibaldi and Perrier, 1975; van Rossum, 1971; van Rossum et al., 1977; Wagner, 1971, 1976; van Ginneken, 1976). When exclusively first-order processes are considered the plasma concentration of a drug can be written as a function of time in an integrated form. For any linear n-compartment model the plasma concentration curve can be fitted to a sum of exponential terms (Reversely, however, this does obviously not prove that a compartment model is involved. Stochastic processes may lead as well to a sum of exponentials). As a matter of fact, the plasma curve depends also on the input function (dose, dosage form). After administration of a single dose, the following general equations are valid:

In case of intravenous injection (pulse input):

\[
C = \sum_{i=1}^{n} A_i e^{-t/\tau_i} \quad \text{(Eq. 1.1)}
\]

In case of intravenous infusion during 0-T (step function):

\[
C = \sum_{i=1}^{n} A_i \cdot \tau_i / T \ (1-e^{-t/\tau_i}) \quad \text{for } t \leq T \quad \text{(Eq. 1.2a)}
\]

and

\[
C = \sum_{i=1}^{n} A_i \cdot \tau_i / T \ (e^{-(t-T)/\tau_i} - e^{-t/\tau_i}) \quad \text{for } t \geq T \quad \text{(Eq. 1.2b)}
\]

In case of oral administration (first-order input)

\[
C = \sum_{i=1}^{n} A_i \left( e^{-t/\tau_i} - e^{-t/\tau_a} \right) \quad \text{(Eq. 1.3)}
\]
In these equations $C$ represents the concentration in plasma, $t$ is the time after the start of the infusion or after intake of the oral dose, $T$ is the duration of the infusion, $\tau_i$ are the time constants corresponding to the various decay phases visible in a semi-logarithmic plot of the plasma concentration against time ($i = 1 \ldots n$; the largest time constant is equal to 1.44 times the terminal half-life), and $\tau_a$ is the time constant of first-order absorption into the central compartment. In case of i.v. administration, the coefficients $A_i$ are the hypothetical intercepts with the ordinate for an intravenous bolus injection of the same amount of drug. The intercepts after oral administration ($A_i^{PO}$) and those after i.v. administration ($A_i^{IV}$) are for equal doses related as follows: $A_i^{PO} = A_i^{IV} \cdot \frac{\tau_i}{(\tau_i - \tau_a)}$.

The search for the parameters $A_i$ and $\tau_i$ best fitting to the experimental concentration-time data is facilitated by a suitable computer curve-fitting program. The weighed least-squares regression analysis program FARMFIT, used in our investigations, approaches the model function as a set of linear ($A_i$) and non-linear ($\tau_i$) parameters. Non-linear parameter estimates are optimized by means of an iterative Gauss-Newton procedure and the linear parameters are calculated analytically. A full description of the FARMFIT program has been given elsewhere (Breimer, 1974) and a listing of the main routine and current subroutines is available (Klok, 1979). Once the constants $A_i$ and $\tau_i$ have been found, they may serve to calculate directly two important pharmacokinetic parameters: the plasma clearance ($k_{Cel}$) and the total volume of distribution at steady-state ($V_{dss}$) (van Rossum, 1971; Wagner, 1976):

In case of intravenous administration:

$$k_{Cel} = \frac{D}{n \sum_{i=1}^{n} A_i \cdot \tau_i}$$  \hspace{1cm} (Eq. 1.4)

and

$$V_{dss} = \frac{D \sum_{i=1}^{n} A_i \cdot \tau_i^2}{(\sum_{i=1}^{n} A_i \cdot \tau_i)^2}$$  \hspace{1cm} (Eq. 1.5)
In case of oral administration:

\[
\begin{align*}
k_{\text{Cel}} &= \frac{F \cdot D}{\sum_{i=1}^{n} \left(1-\frac{\tau}{\tau_i}\right) \cdot A_i \cdot \tau_i} \quad \text{(Eq. 1.6)} \\
V_{\text{dss}} &= \frac{F \cdot D \sum_{i=1}^{n} \left(1-\frac{\tau}{\tau_i}\right) \cdot A_i \cdot \tau_i}{\left(\sum_{i=1}^{n} \left(1-\frac{\tau}{\tau_i}\right) \cdot A_i \cdot \tau_i\right)^2} \quad \text{(Eq. 1.7)}
\end{align*}
\]

where D denotes the dose, and F the fraction of dose coming into the general circulation after oral administration. The plasma clearance \((k_{\text{Cel}})\), which equals the total body clearance if the elimination of the drug occurs from the central compartment only, is in fact an abstract parameter. It represents that volume of fluid which is entirely cleared of drug per unit time. The clearance concept is very useful because it provides a link between pharmacokinetics and the underlying physiological processes, e.g. the capacity of liver enzymes to metabolize foreign compounds or that of the kidney to excrete them (van Rossum et al., 1977). Thus, for instance, the total plasma clearance of a hypothetical drug that is not metabolized and only excreted by the kidneys will in general not be able to exceed the renal plasma flow.

The volume of distribution \((V_{\text{dss}})\) is defined as that hypothetical volume over which the total amount of drug in the body would be distributed at steady-state if we assumed that this volume would contain a concentration equal to that measured in plasma. Because in reality very unequal tissue over plasma concentration ratios occur, it will be clear that this volume is a purely operational parameter: a large \(V_{\text{dss}}\) implies low plasma concentrations and extensive localization of the drug outside the plasma.

The equations 1.1 - 1.7 can also be formulated in terms of rate constants instead of their reciprocals - the time constants - (cf. e.g. Chapter 9). Although both ways of expression are mathematically entirely equivalent, there are certain arguments in favour of the time constants in relation to...
the concept of the mean residence time of a drug (van Rossum, 1979). The mean residence time (τ) is defined as:

\[ \tau = \int_{0}^{\infty} t \cdot C \, dt / \int_{0}^{\infty} C \, dt, \]

which is equivalent to

\[ \sum_{i=1}^{n} A_i \cdot \tau_i^2 / \sum_{i=1}^{n} A_i \cdot \tau_i. \]

It follows then from the equations 1.4 and 1.5 that \( V_{dss} = \tau \cdot k_{cel} \).

Until now, however, preference is given in current pharmacokinetic literature to the notation with rate constants. We will conform to this convention in the following chapters (see e.g. Chapter 9), so that a rapid comparison can be made of the pharmacokinetic parameters from our studies with those from the literature.

No explicit expression for the plasma concentration as a function of time can be derived in general if non-linear, i.e. not first-order, processes are involved. Non-linear pharmacokinetics considers capacity-limited clearance pathways and/or saturable tissue binding phenomena. Only for a very simple model an integrated form of the model equation can be obtained (van Ginneken et al., 1974), but in other cases use has to be made of differential equations to describe the individual steps of drug transport between compartments. Solutions can then be found numerically by means of a computer program such as FARMFIT (e.g. van Rossum et al., 1977; Fleuren and van Rossum, 1977; Chapter 7).

The aim of any pharmacokinetic model should be more than an efficient reduction of experimental data to a few essential parameters. Insight should be obtained in the underlying biological processes. For this purpose, human kinetic studies always need additional information derived from, for instance, whole-body distribution studies in animals. Attempts to integrate real physiological entities like organ blood flows and tissue over plasma concentration ratios of drugs to a multicompartment drug disposition model have been described (Bischoff et al., 1971; Dedrick, 1973; Harrison and Gibaldi, 1977; Chen and Gross, 1979.) Such system-dynamic approach seems very promising but requires exact filling-in of all individual elements. Until that situation has been accomplished, the compartment model concept and related parameters as clearance and volumes of distribution can be assumed to remain most adequate for an overall characterization of the processes of absorption, distribution and elimination of drugs.
HUMAN PHARMACOKINETICS OF SULFONAMIDE DIURETICS

Despite the bewildering number of sulfonamide diuretics available since 1960 pertinent information on the pharmacokinetics of these drugs is barely at hand. As is outlined in Chapter 2 this may be ascribed to the use of unselective methods of analysis in the earliest investigations, while specific and sensitive assays were only recently developed. In this context, Benet (1979) has addressed himself to the problem of conflicting kinetic data reported on furosemide and concluded that part of these discrepancies was due to unspecificity of the older analytical methods.

Volumes of distribution

Those data from normal humans available today, which were selected from the literature under the condition that they were based on methods of determination specific for the parent drugs, are presented in Table 1.2*. Two subgroups can be discerned. Compared with the thiazides the loop diuretics bumetanide and furosemide have relatively small volumes of distribution. This can readily be explained from the $pK_a$ difference between these two drugs and the other sulfonamide diuretics (see Chapter 2). So, at physiological pH bumetanide and furosemide are largely in anionic form which will limit their passage across cell membranes. The finding that both drugs accumulate less than the thiazides in proximal tubular cells (Peters and Roch-Ramel, 1969b; Duggan, 1966; Cohen et al., 1976) indicates that this physico-chemical characteristic is certainly relevant, notwithstanding the occurrence of active tubular secretion for all sulfonamide diuretics. Moreover, furosemide and bumetanide are not stored in liver cells to the extent known from the thiazides (Pulver et al., 1959; Taugner and Iravani, 1965; Peters and Roch-Ramel, 1969a; Duhm et al., 1967). It has been reported also that bumetanide does not enter into red blood cells (Pentikainen et al., 1977) and the same would be expected to occur for furosemide.

Uptake in red blood cells

Besides the accumulation of the thiazides - including chlorthalidone and mefruside - in kidney and liver tissue, these drugs are taken up also by red blood cells to a variable extent. This factor obviously makes another contribution to establish a large apparent volume of distribution, if its calculation is based on plasma concentration measurement. Thus, after administration of single doses of these drugs to humans, an erythrocyte over plasma concentration ratio of ca. 2.5 has been reported for hydroflume-

* This part of the manuscript was completed 1st May, 1979.
<table>
<thead>
<tr>
<th>Generic name b</th>
<th>Total volume of distribution (l)</th>
<th>Plasma clearance (l/h)</th>
<th>Plasma clearance (ml/min)</th>
<th>Renal clearance (% of total plasma clearance)</th>
<th>Terminal t(\frac{1}{2}) (h)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bumetanide</td>
<td>ca. 12.5</td>
<td>ca. 12</td>
<td>ca. 200</td>
<td>~ 60</td>
<td>1.2-1.8</td>
<td>c</td>
</tr>
<tr>
<td>(Burinex®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td>12.5-15</td>
<td>ca. 12</td>
<td>ca. 200</td>
<td>~ 60</td>
<td>0.8-1.2</td>
<td>d</td>
</tr>
<tr>
<td>(Lasix®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td>≥ 100</td>
<td>≤ 20</td>
<td>≤ 350</td>
<td>≥ 30</td>
<td>≥ 2.4-3.8</td>
<td>e</td>
</tr>
<tr>
<td>(Pluryl®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>200-350</td>
<td>5-9</td>
<td>80-150</td>
<td>~ 70</td>
<td>25-50</td>
<td>f</td>
</tr>
<tr>
<td>(Hygroton®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>ca. 250</td>
<td>ca. 12.5</td>
<td>ca. 200</td>
<td>&gt; 90</td>
<td>13.6</td>
<td>g</td>
</tr>
<tr>
<td>(Dichlotride®, Esidrex®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroflumethiazide</td>
<td>ca. 250</td>
<td>ca. 12.5</td>
<td>ca. 200</td>
<td>&gt; 90</td>
<td>12.5-25</td>
<td>h</td>
</tr>
<tr>
<td>(Rontyl®, Hydroflumethiazidum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mefruside</td>
<td>300-500</td>
<td>20-135</td>
<td>350-2250</td>
<td>&lt; 1</td>
<td>2.9-12.5</td>
<td>i</td>
</tr>
<tr>
<td>(Baycaron®)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active metabolites of mefruside</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt; 60</td>
<td>10-14</td>
<td>i</td>
</tr>
</tbody>
</table>

a Taken or recalculated from the references indicated. Some of these contain only partial information on a particular drug.
bNames of proprietary products marketed in the Netherlands in parentheses.
cDavies et al., 1974; Halladay et al., 1977, Pentikainen et al., 1977.
eBeermann et al., 1977c. Note: plasma concentration followed only for 10 hr.
fFleuren et al., 1979a; Chapter 9.
gBeermann et al., 1976. Note: sufficient data for calculation from 1 subject only.
hBrors et al., 1978.
iFleuren et al., 1979c; Chapter 14. Note: parameters calculated under assumption of complete oral bioavailability.
thiazide (Brors et al., 1978), a ratio of ca. 3.5 for hydrochlorothiazide (Beermann et al., 1976), a ratio of 20-40 for mefruside (Fleuren et al., 1979c; Chapter 14) and a ratio of 50-100 for chlorthalidone (Collste et al., 1976; Beermann et al., 1975b; Fleuren and van Rossum, 1975, 1977; Fleuren et al., 1979a, 1979b; Chapters 7, 9 and 10; Riess et al., 1977). As all sulfonamide diuretics are in vitro inhibitors of red cell carbonic anhydrase, though to a weaker extent than acetazolamide (Beyer and Baer, 1961; Meng and Loew, 1974), binding to this intracellular protein might be involved. As is discussed in Chapter 8, this has definitely been established in case of chlorthalidone, while some evidence for binding of mefruside to red blood cell carbonic anhydrase is given in Chapter 14. Interestingly, the red cell over plasma concentration ratio of the four above-mentioned compounds increases in exactly the same order as the carbonic anhydrase inhibitory potency of these drugs (the actual drug concentrations at 50% inhibition can be found in the reports by Beyer and Baer (1961) and Meng and Loew (1974)).

**Plasma protein binding**

Not only binding to intracellular components but also the extent of plasma protein binding determines of course such a distribution ratio. The red cell over plasma concentration ratio in the above series of compounds can, however, be expected to be a true reflection of the extent of red blood cell binding if plasma protein binding would be the same. This appears roughly to be the case. Hydroflumethiazide is for 75% bound to human serum albumin (Agren and Bäck, 1973), chlorthalidone is also for 75% protein-bound (Collste et al., 1976; Dieterle et al., 1976). For mefruside 70-80% binding has been reported to plasma proteins of dogs (Schlossman, 1967). No data are available for hydrochlorothiazide, but due to its structural similarity to hydroflumethiazide a similar figure may be anticipated.

The other sulfonamide compounds appearing in Table 1.2 occur even more protein-bound in human plasma. For bumetanide a figure of 96% has been reported (Pentikainen, 1977), for furosemide 96-98% (Andreasen and Mikkelsen, 1977; Andreasen et al., 1978) and for bendroflumethiazide 94% (Agren and Bäck, 1973).

**Glomerular filtration, tubular secretion and reabsorption**

A high plasma protein binding means that the fraction of drug available for glomerular filtration is very low, as it may be assumed that only unbound drug can be filtered into the proximal renal tubules. Therefore, the maximum renal clearance that would theoretically be possible if glomerular filtration were the only route of elimination would equal glomerular filtration rate (120 ml/min for a normal human subject) times the free fraction of each drug. From Table 1.2 it can be seen that the actual renal clearance values of most drugs are much larger, which confirms the presence of an
additional way of elimination, i.e. tubular secretion by the organic acid transport system described in a previous section of this chapter.

So, a renal clearance higher than that attributable to glomerular filtration alone is a prima facie argument for tubular secretion. The absence of such a clear circumstance, however, does not allow the conclusion that no secretion is involved because of the possibility that the net renal excretion of a drug is diminished by reabsorption into the blood capillaries around the renal tubules. The probability of such a process would be expected to increase with increasing lipophilicity in a series of chemically related substances. Cyclopenthiazide and polythiazide (Fig. 1.1) are very lipophilic benzothiadiazines (Schlossmann, 1974) and the older literature suggests that their renal clearances are smaller than those of the more hydrophilic compounds (Peters and Roch-Ramel, 1969a). However, as mentioned in the first passage of this section, unspecific and also highly unsensitive methods (see Chapter 2) were used for these studies, so that exact interpretation is difficult. The same caution is in place with respect to recent data from Hobbs and Twomey (1978), who developed a very sensitive GLC assay for polythiazide, the specificity of which could, however, not be warranted. Nevertheless, the rather small renal plasma clearance of about 30 ml/min which can be calculated for polythiazide from their data could be in agreement with occurrence of tubular reabsorption of this relatively lipophilic diuretic. For mefruside, which has an even larger ether-water partition coefficient than polythiazide and cyclopenthiazide (Schlossmann, 1974), a very low renal plasma clearance, 2.5-5 ml/min, has been established (Fleuren et al., 1979c; Chapter 14). Further support for reabsorption in more distal parts of the nephron can be borrowed from a slight dependence of the renal clearance of several thiazides including chlorthalidone upon urine pH (Beyer and Baer, 1975; Chapter 10).

**Plasma clearance**

Contrary to the difference in the apparent total volume of distribution, the total plasma clearance of furosemide and bumetanide does not differ markedly from that shown by the diuretics of the thiazide group (Table 1.2). So, the conclusion seems justified that short half-lives of diuretics are just the consequence of small volumes of distribution and not due to a large affinity for renal (and non-renal) elimination mechanisms.

The plots of plasma concentration and urinary excretion rate against time were reported to run parallel for all drugs quoted in Table 1.2. This implies that no saturation of the tubular transport system has become apparent after single doses of these drugs (such statement cannot be made, however, about the kinetics during chronic administration, because sufficient data about this situation have not yet been published). The linear relationship between the plasma concentration and urinary excretion rate
of diuretics in single dose studies might constitute an analytical advantage. As is outlined in Chapter 2, the concentrations of the thiazide diuretics in urine are very high compared to those in plasma, so that future determination of individual half-lives of these drugs may be performed much more easily by assay of urine instead of plasma.

**Pharmacokinetics and diuretic effects**

The half-life values of the diuretics quoted in Table 1.2 are approximately proportional to the duration of the natriuretic effects after single doses of these drugs. So, furosemide and bumetanide are known as short-acting diuretics with an effect that is over after 3-4 hours, bendroflumethiazide acts for about 10 hours, chlortalidone for up to 48-72 hours, and the effects of hydrochlorothiazide, hydroflumethiazide and mefruside last during 12-24 hours (Beermann et al., 1977c; Brors et al., 1977; Davies and Wilson, 1975). Mefruside shows considerable intersubject variation in elimination half-life, but forms metabolites which must be considered to be responsible for its diuretic activity (Fleuren et al., 1979c; Chapters 14 and 15). As is outlined before in this Chapter, the natriuretic effect of sulfonamide diuretics appears to correlate better with the amount of drug reaching the urine than with the height of the plasma concentration. This conclusion is supported by the observation that all diuretic drugs, for which at present sufficient data are available, are characterized by a considerable extent of urinary excretion. A survey of the literature is presented in Table 1.3. This table contains only those figures, which were based upon analytical methods which assayed the unchanged drugs specifically. (So, we assumed that these species represented the active principles and, therefore, would be relevant for a correlation with diuretic effect.) For this reason, data on, for instance, chlorothiazide after intravenous administration, which indicated that all radioactivity was excreted into human urine (Brettell et al., 1960), were not included because the reported method (Beyer, 1958; Brettell et al., 1960) did not sufficiently justify the assumption that unchanged drug alone had been analysed. Similarly a definite range of half-life values of hydrochlorothiazide could not yet be given in Table 1.3, because there is reason to suspect that the actual t½ is longer than most figures available to date. Thus, usually a t½ value of 3-5 hours has been reported for hydrochlorothiazide (Meyer and Whyatt, 1976; Cooper et al., 1976), but incidentally, when analytical measurement was sufficiently sensitive, t½ values tended to be larger, up to 15 hours (Beermann and Groschinsky-Grind, 1977; Beermann et al., 1976; Redalieu et al., 1978). For most drugs quoted in Table 1.3 the length of the assay period is sufficiently in excess of the half-life. So, the cumulative urinary amounts shown must be a reasonable approximation of the total amounts excreted at infinite time.
<table>
<thead>
<tr>
<th>Drug name</th>
<th>Dose and mode of administration</th>
<th>Elimination half-life (h)</th>
<th>Urine assay period</th>
<th>Cumulative amount excreted unchanged (% of dose)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bendroflumethiazide</td>
<td>10 mg, p.o. (9)</td>
<td>2.4-3.8</td>
<td>48 h</td>
<td>21-37</td>
<td>d</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>0.5 mg, i.v. (4)</td>
<td>1.3-1.7</td>
<td>6 h</td>
<td>ca. 60</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>0.5 mg, p.o. (4)</td>
<td>1.2-1.6</td>
<td>6 h</td>
<td>ca. 50</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>1 mg, p.o. (6)</td>
<td>24 h</td>
<td>ca. 45</td>
<td></td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>2 mg, p.o. (3)</td>
<td>24 h</td>
<td>50-60</td>
<td></td>
<td>g</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>50 mg, i.v. (7)</td>
<td>25-50</td>
<td>∞</td>
<td>50-78</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>50 mg, p.o. (6)</td>
<td>43-51</td>
<td>∞</td>
<td>32-51</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>50 mg, p.o. (6)</td>
<td>37-59</td>
<td>192 h</td>
<td>41-63</td>
<td>j</td>
</tr>
<tr>
<td>Furosemide</td>
<td>40 mg, i.v. (6)</td>
<td>—</td>
<td>24 h</td>
<td>38-88</td>
<td>k</td>
</tr>
<tr>
<td></td>
<td>4-10 mg, p.o. (2)</td>
<td>—</td>
<td>24 h</td>
<td>ca. 30</td>
<td>l</td>
</tr>
<tr>
<td></td>
<td>7 mg, i.v. (2)</td>
<td>—</td>
<td>3 h</td>
<td>ca. 50</td>
<td>l</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>1.35 mg, i.v. (2)</td>
<td>—</td>
<td>4-7 d</td>
<td>ca. 90</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>12.5-75 mg, p.o. (8)</td>
<td>—</td>
<td>48 h</td>
<td>65-72</td>
<td>n</td>
</tr>
<tr>
<td>Hydroflumethiazide</td>
<td>150 mg, p.o. (5)</td>
<td>12.4-26.9</td>
<td>∞</td>
<td>41-59</td>
<td>o</td>
</tr>
<tr>
<td>Mefruside metabolites</td>
<td>50 mg, p.o. (4)</td>
<td>10-14</td>
<td>∞</td>
<td>48-67</td>
<td>p</td>
</tr>
<tr>
<td>Metolazone</td>
<td>2.5 mg, i.v. (6)</td>
<td>—</td>
<td>144 h</td>
<td>ca. 70</td>
<td>q</td>
</tr>
<tr>
<td>Xipamide</td>
<td>42 mg, i.v. (5)</td>
<td>—</td>
<td>24 h</td>
<td>50-60</td>
<td>r</td>
</tr>
</tbody>
</table>

>a Number of subjects in parentheses; b Based upon plasma concentration in all cases, except for hydroflumethiazide and mefruside-metabolites (urine assay); c ∞ indicates at infinite time, calculated by extrapolation of actual data; d Beermann et al., 1977c; e Pentikainen et al., 1977; f Peit et al., 1973; g Halladay et al., 1977; h Fleuren et al., 1979a; Chapter 9; i Fleuren et al., 1979b; Chapter 10; j Riess et al., 1977; k Andreasen and Mikkelsen, 1977; l Beermann et al., 1975a; m Beermann et al., 1976; n Beermann and Groschinsky-Grind, 1977; o Brors et al., 1978; p Fleuren et al., 1979c; Chapter 14; q dose administered as mefruside; r Tilstone et al., 1974; s Hempelman and Dieker, 1977
The qualitative effect of a drug is determined by its interaction with a receptor in the target organ. The intensity and duration of that effect are modified by pharmacokinetic factors. The sulphonamide diuretics chlorthalidone and mefruside were selected for the present studies, because they are used by large numbers of patients in antihypertensive therapy, while very little was known about the disposition of these drugs in man. As discussed in this introductory chapter, some general insight has now been acquired in the relationship between the pharmacokinetics and the pharmacodynamics of sulphonamide diuretics. For this purpose, literature data on the kinetics of some benzothiadiazines and furosemide and bumetanide were compared with the observations, described in the following chapters of this thesis.

Prior to these pharmacokinetic studies considerable efforts were made, as evident from section II, to realize selective and sensitive methods for the assay of chlorthalidone, mefruside and its metabolites in human body fluids. The circumstance that both drugs bind to a large extent to erythrocytes has made high demands to the analytical methodology, because no artifacts in the distribution between red cells and plasma should be introduced.

Section III deals with the human kinetics of chlorthalidone. The main issues of interest are: the time course of plasma concentration, red blood cell concentration, and urinary excretion rate both after single and multiple doses, furthermore the contribution of the routes of renal and biliary excretion, and metabolism, to overall elimination, and the absolute bioavailability of oral doses as determined by reference to the intravenous dosage form.

Section IV describes the pharmacokinetic characteristics of mefruside in man. Much attention is paid to two of its metabolites, because there are good reasons to assume that these metabolites constitute the really active principles of mefruside.

It is hoped that the studies, described in this thesis, will provide a reference for future work with the above substances as well as other diuretic drugs.
REFERENCES

Agren, A. Back, R: Complex formation between macromolecules and drugs. VII. Binding of saccharin, N-methylsaccharin, and the diuretic drugs hydroflumethiazide and bendroflumethiazide to human serum albumin. *Acta Pharm. Suec* 10, 223-228 (1973)


Beisenherz, G., Koss, F.W., Klatt, L., Binder, B: Distribution of radioactivity in the tissues and excretory products of rats and rabbits following administration of Cl4-Hygroton. *Arch. int. Pharmacodyn.* 161, 76-93 (1966)


Dost, F.H : Der Blutspiegel Leipzig. Thieme 1953


Wagner, J. G.: Linear pharmacokinetic equations allowing direct calculation of many needed pharmacokinetic parameters from the coefficients and exponents of polyexponential equations which have been fitted to the data. J. Pharmacokin. Biopharm 4, 443-467 (1976).


GENERAL INTRODUCTION TO ANALYSIS OF DIURETIC DRUGS IN BIOLOGICAL FLUIDS

INTRODUCTION

Many drugs cannot be assayed directly in the biological fluid, due to interferences by numerous endogenous substances. A commonly employed purification step involves extraction from the aqueous phase into a suitable organic solvent. Distribution of weak acids and bases between organic and water phases is governed, besides by lipophilicity, by the degree of ionization of the compound under study. Therefore, the acid or base dissociation constant (pKa) is an important parameter in partitioning studies. The pKa values of the frequently used sulfonamide diuretics range between 3.6 and 9.5, as can be seen from the survey of Table 2.1. Apart from furosemide and bumetanide, which contain each a carboxylic acid moiety, most thiazides possess two weakly acidic sulfonamide groups. In order to distinguish one from the other, Mollica et al. (1971) replaced the ring-sulfonamide proton of hydrochlorothiazide by an ethyl group and observed for that compound only one pKa, at 9.5. Obviously, therefore, the lower pKa value belonged to the sulfonamide proton in the thiazide ring. In agreement with this, chlorthalidone and mefruside, which possess only the free sulfamoyl group, showed pKa values above 9, see Table 2.1.

As a consequence, most sulfonamide diuretics including chlorthalidone and mefruside, except the first three of the survey of Table 2.1, can be extracted up to high pH values. See e.g. the plot of extraction recovery of chlorthalidone vs pH of the aqueous phase in Fig. 2.1. It should be noticed from this plot that the pH value where half the maximal amount of drug is extracted does not coincide with the pKa value of that drug. The curve has been shifted to the right because of contributions from the lipophilicity of the drug and the ratio of the phase volumes used:

$$pH_{0.5} \approx pKa + \log \left( \frac{TPC \cdot V_{org}}{V_{aq}} - 1 \right)$$

In this equation, derived in Appendix 2.1 at the end of this chapter, pH0.5 denotes the pH at which half maximal extraction recovery occurs, TPC the true partition coefficient of the drug, and Vorg and Vaq represent the volumes of the organic and aqueous phase respectively. The TPC depends on the choice of the organic solvent, so the TPC = 35 when partitioning of
<table>
<thead>
<tr>
<th>Drug name</th>
<th>Structural formula</th>
<th>( pK_{a1} )</th>
<th>( pK_{a2} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bumetanide</td>
<td><img src="image" alt="Structural formula for Bumetanide" /></td>
<td>3.6</td>
<td>7.7</td>
<td>Orita et al., 1976</td>
</tr>
<tr>
<td>Furosemide</td>
<td><img src="image" alt="Structural formula for Furosemide" /></td>
<td>3.8</td>
<td>7.5</td>
<td>Orita et al., 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.9</td>
<td></td>
<td>Hajdú and Haussler, 1964</td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td><img src="image" alt="Structural formula for Chlorothiazide" /></td>
<td>6.7</td>
<td>9.5</td>
<td>Charnicki et al., 1959</td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td><img src="image" alt="Structural formula for Bendroflumethiazide" /></td>
<td>8.5</td>
<td></td>
<td>Agren and Bäck, 1973</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td><img src="image" alt="Structural formula for Hydrochlorothiazide" /></td>
<td>8.6</td>
<td>9.9</td>
<td>Mollica et al., 1971</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.7</td>
<td></td>
<td>Mollica et al., 1971</td>
</tr>
<tr>
<td>Hydroflumethiazide</td>
<td><img src="image" alt="Structural formula for Hydroflumethiazide" /></td>
<td>8.9</td>
<td>10.5</td>
<td>Smith et al., 1976</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td><img src="image" alt="Structural formula for Chlorthalidone" /></td>
<td>9.35</td>
<td>(~11)</td>
<td>Fleuren et al., 1979c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chapter 6</td>
</tr>
<tr>
<td>Mefruside</td>
<td><img src="image" alt="Structural formula for Mefruside" /></td>
<td>9.5</td>
<td></td>
<td>Schlossmann, 1974</td>
</tr>
</tbody>
</table>

\(^{a}\)Determined at 25°C by various methods. Original references should be consulted.
Fraction of chlorthalidone in organic phase after equilibration with equal volumes of aqueous buffers of varying pH (citrate-phosphate-borate, 0.1 M, Teorell and Stenhagen). Computer fitting according to Eq. 2.9 yielded a TPC = 35 by use of methyl isobutyl ketone and a TPC = 1.5 by use of diethylether.

Chlorthalidone is carried out with methyl isobutyl ketone, whereas the TPC = 1.5 if diethylether is employed (calculation of TPC by use of Eq. 2.9 in Appendix 2.1). Therefore, when an appropriate organic solvent has been chosen, almost complete extraction recovery of diuretic drugs can be achieved at a pH just below their pKa value. An extraction pH around 8 is more advantageous than a lower one, because it avoids co-extraction of endogenous substances which are often of acidic nature (e.g. fatty acids) and of many acidic drugs.

SEPARATION AND DETECTION OF DIURETIC DRUGS: REVIEW AND DISCUSSION

Detection methods without chromatographic separation

The first methods of assay of the modern thiazide diuretics came into use around 1960 and were based upon the Bratton-Marshall reaction for primary aromatic amines (Bratton and Marshall, 1939). After organic sol-
vent extraction, the thiazides were hydrolysed by acid or alkali to liberate the amino group, followed by diazotation with nitrous acid and coupling with N-(1-naphtyl)ethylenediamine to yield a red colour. Such colorimetric analyses have been reported for, for instance, chlorothiazide (Baer et al., 1959), hydrochlorothiazide (Sheppard et al., 1960a), furosemide (Hajdú and Haußler, 1964) and mefruside, the latter after substitution of an amino group for its chlorine atom (Schlossmann, 1967). Although these methods were of value for obtaining a first impression of the relative importance of the various routes of elimination of these diuretic agents, very high doses, i.e. far above the therapeutic amounts, had to be administered in order to reach measurable blood levels (above ca. 10 μg/ml.). Besides this apparent unsensitivity, unselectivity too, e.g. with regard to discrimination between the parent drug and its possible metabolites, obviously forms a limitation for use of this method in detailed pharmacokinetic studies.

Apart from the above drawbacks, irreproducible colour formation in the Bratton-Marshall reaction due to disturbances by endogenous components of biological fluids has been described (Resetarits and Bates, 1979; Schäfer et al., 1977a).

More sensitive, but remaining questionable with respect to interference by possible drug metabolites is the use of spectrofluorometry, as employed by Smith et al. (1976) and Brors et al. (1977) for the determination of hydroflumethiazide in plasma and urine, and by Hajdú and Haußler (1964) in case of furosemide. The same objection can be raised against the earliest assays of chlorthalidone, based upon ultraviolet absorption of its hydrolysis product (Pulver et al., 1959; Tweeddale and Ogilvie, 1974) and against a later one employing the carbonic anhydrase inhibitory action of this drug (Johnston et al., 1977). Dixon et al. (1976) reported a sensitive radio-immunoassay for bumetanide, and "proved" its specificity by comparison of the concentrations found with the values obtained by plasma radioactivity and ultraviolet absorption measurements, which, however, cannot be regarded as specific assays themselves.

Similarly, pharmacokinetic studies using radioactive-labelled diuretic drugs (Anderson et al., 1961; Brettell et al., 1960, 1964; Calesnick et al., 1966; Rupp et al., 1972; Sheppard et al., 1960) cannot be assumed to have provided specific information on the parent drug as long as no definite proof has been given that no metabolites were included in the assay. Complete or almost complete absence of metabolites appears to be verified until now only in case of administration of hydrochlorothiazide to humans, where more than 95% of the dose was eliminated in unchanged form (Beermann et al., 1976b). An identical situation may be expected for hydroflumethiazide, owing to its great structural resemblance to hydrochlorothiazide, see Table 2.1. The data on this drug, available so far,
indicated that metabolite formation accounted for not more than ca. 2% of the dose (Brors et al., 1978), which is in agreement with the above assumption. For other sulfonamide diuretics, however, no such pertinent information is present. On the contrary, our investigations with intravenously administered chlorthalidone indicate that this drug is metabolized in man for about 30% of the dose administered (Fleuren et al., 1979a; Chapter 9, this thesis). A still larger percentage has been found for bendroflumethiazide by Beermann et al. (1977). Only when the structure of the metabolites has become known, it shall be possible to determine their influence on the analytical results obtained. At that time, several of the methods mentioned above, if suitably adapted, might appear very convenient for the purpose of pharmacokinetic studies. Until then, however, specific methods involving separate determination of the parent drug should be used. The next sub-sections will deal, therefore, with separation techniques, commonly employed for the assay of diuretics in body fluids, and the advantages and limitations of these methods will be indicated.

**Thin-layer chromatography**

Thin-layer chromatography (TLC) was the earliest chromatographic technique by which diuretics occurring in urine could be separated, with the use of ultraviolet absorption, iodine vapour and various spray reagents as detection tools (e.g. Maes et al., 1970; Osborne, 1972; Sohn et al., 1973). Only semi-quantitative information could be gathered, because the concentrations encountered were only slightly above the analytical limits of detection: depending upon the structure of the particular diuretic and upon the way of detection, absolute amounts on the plate down to 0.5-5 µg could be visualized.

Improvement in sensitivity was obtained by using spectrofluorometry to determine hydroflumethiazide (Brors et al., 1978; Garceau et al. 1974), hydrochlorothiazide (Schäfer et al., 1977a) and furosemide (Schäfer et al., 1977b) down to concentrations of 20-50 ng/ml in plasma.

**High-pressure liquid chromatography**

When we look at the polar groups of the sulfonamide diuretics, some of which have been depicted in Table 2.1, high-pressure liquid chromatography (HPLC, alternatively called high-performance liquid chromatography), seems at first glance the technique of choice for determination of such compounds. HPLC works particularly well in the determination of diuretic drugs in pharmaceutical dosage forms (Butterfield et al., 1978; O’Hare et al., 1979; Wong et al., 1977). Also urine concentrations of diuretics after therapeutic doses can be readily assayed, as evidenced by HPLC methods for hydrochlorothiazide (Cooper et al., 1976), chlorothiazide (Hunt et al., 1978; Lin and Benet, 1978; Resetarits and
Bates, 1979), furosemide (Carr et al., 1978; Lindström, 1974), 5-oxo-
mefruside (Little et al., 1977), bemetizide (Brodie et al., 1978) and
metolazone (Hinsvark et al., 1972).

Plasma concentrations of most diuretic drugs, however, are much lower
than the corresponding urine concentrations, as discussed in the con­
cluding remarks section below. Published work dealing with assay of
diuretics in this biological fluid indicates that at best concentrations down
to ca. 50 ng/ml can be measured accurately, the actual levels depending on
the ultraviolet absorptive properties of the diuretics investigated (Brodie et
al., 1978; Carr et al., 1978; Christophersen et al., 1977; Cooper et al.,
1976; Lindström, 1974; Nation et al., 1979).

Gas chromatography

The main difficulty in applying gas chromatography (GC) to the assay of
sulfonamide diuretics was that volatile derivatives of these compounds had to
be prepared. In case of hydrochlorothiazide, neither silylation nor methyl­
atation with diazomethane gave complete derivative formation (Vandenheuvel
et al., 1975). The same results were found during our preliminary analytical
work with chlorthalidone (unpublished observation). Similar findings, also
with respect to acetylation, were reported by Li et al. (1977).

Flash-heater methylation with trimethylanilinium hydroxide, according
to Brochmann-Hanssen and Oke (1969), led to complete conversion of
both hydrochlorothiazide (Vandenheuvel et al., 1975) and chlorthalidone
(Li et al., 1977) to their methyl derivatives. These authors admitted,
however, that the molar ratio of methylation reagent over drug amount
should be kept between rather narrow limits for successful methylation; in
addition, the injector geometry of the gas Chromatograph proved to be a
very critical factor.

Most promising results have been obtained by means of extractive
methylation. This technique, which allows better control of derivatization
conditions than the flash-heater methylation, e.g. with regard to the reac­tion
time, is relatively new in derivative formation for GC and will be
described, therefore, in a separate section of this chapter. GC determina­
tions of the following diuretic drugs all employed permethylation by this
method: chlorthalidone (Ervik and Gustavii, 1974; Degen and Schweizer,
1977; Fleuren and van Rossum, 1978; this thesis, Chapter 3), furosemide
(Lindström and Molander, 1974), hydrochlorothiazide (Lindström et al.,
1975; Redalieu et al., 1978), bendroflumethiazide (Beermann et al., 1976a)
and mefruside (Fleuren et al., 1979b; this thesis, Chapter 4). Extractive
methylation of the above drugs was recently re-established in a comprehen­
sive study by Fagerlund et al. (1979). These authors reported in addition
that the technique had successfully been applied to seven other
sulfonamide diuretics, including bumetanide and cyclopenthiazide.
Besides the disadvantage that volatile derivatives have to be prepared for gas chromatography stands the advantage that sensitive and selective detectors are available. The electron capture detector (E.C.D.) has become the most widely used detector in gas chromatography after the conventional flame ionization detector. With this selective detector numerous analyses of pharmaceutical compounds have been performed, either directly or after derivatization with electron-capturing groups, see e.g. the review by Ahuja (1976). Accurate determination of hydrochlorothiazide and bendroflumethiazide with an E.C.D. was possible down to concentrations of 5-10 ng/ml in plasma (Beermann et al., 1976a; Lindström et al., 1975; Redalieu et al., 1978). Although measurement of even 2 ng/ml of chlorthalidone in plasma was claimed by Ervik and Gustavii (1974), routine experience by other workers indicated that this statement was somewhat too optimistic and that, more realistically, determinations down to ca. 10-20

![Gas chromatograms obtained after assay of low nanogram concentrations of chlorthalidone in plasma by means of a nitrogen-sensitive detector (chl. = chlorthalidone, i.st. = standard).](image-url)
ng/ml were feasible by E.C.D. (Degen and Schweizer, 1977). However, the E.C.D. was not very sensitive towards furosemide, of which minimum plasma concentrations of 0.1 μg/ml could be assayed (Lindström and Molander, 1974).

Another sensitive and selective detector in gas chromatography is the thermionic detector using an alkali salt (nitrogen detector, phosphorus detector, alkali flame ionization detector). Because this detector, which in the nitrogen-mode selectively records nitrogen-containing organic substances, is still less in common use than the E.C.D., a brief summary of its properties and some of its applications in drug analysis will be given separately in one of the following sections of this chapter. Concentrations of both chlorthalidone and mefruside down to 5-10 ng/ml in plasma and urine were assayed with the nitrogen detector, see, e.g., Fig. 2.2 (Fleuren and van Rossum, 1978; Fleuren et al., 1979b; this thesis, Chapters 3 and 4).

Concluding remarks

Obviously, a specific assay of the unchanged drug is a first requirement in drug disposition studies. Because the plasma is the body fluid, via which all transport and exchange processes are mediated, concentration measurements in that central fluid are undispensable to obtain insight in the primary determinants of handling of drugs by the body, like clearance and volumes of distribution. For fundamental pharmacokinetic studies, therefore, a sensitive assay in plasma is needed too. Many diuretic drugs are excreted into urine as the unchanged compound for a considerable part of the dose (Chapter 1), implying that relatively high urinary concentrations occur (range ca. 0.1-15 μg/ml), see for instance Fig. 2.3. The total volume of distribution of these drugs is, however, very high so that after single therapeutic doses very low plasma concentrations result in the terminal part of the concentration-time curves, e.g. from 5-100 ng/ml. This circumstance indicates that the detection limit of the analytical method is critical for obtaining reliable estimates of the terminal plasma half-life of these drugs. So, if plasma concentrations of 5 ng/ml instead of 50 ng/ml can be assayed, additional information will be available over a period that corresponds to ca. three times the terminal half-life of that drug. In the absence of this information erroneous impressions of this biological half-life can be found. This has been demonstrated very clearly in case of hydrochlorothiazide and hydroflumethiazide. While Cooper et al. (1976) reported a mean "half-life" of 5 hr for hydrochlorothiazide, based upon measurement down to ca. 100 ng/ml by HPCL, the t½ values appeared to be much longer, ca. 15 hr, when measurement was performed down to the 10 ng/ml level by GC with E.C.D. (Beermann et al., 1976; Redalieu et al., 1978). Analogously, Brors et al. (1977) reported a "half-life" of ca. 2 hours for hydroflumethiazide, but t½ values of 13-25 hr were
Comparison of urine and plasma concentrations of the diuretic chlorthalidone after a single 100 mg dose in man.

found by the same authors in a later study (Brors et al., 1978) in which they admitted that the values obtained in their earlier communication had not to be regarded as the final half-lives.

These cases may suffice to indicate that the sensitivity required is provided until now only by detectors which are available in gas chromatography. In high-pressure liquid chromatography there is clearly a need for lower limits of detection. The future will have to show us if wider application of e.g. fluorescence or electrochemical detectors can fulfil this need.

HPCL, however, seems already very convenient for the assay of diuretics in urine. In a later phase of a pharmacokinetic study, therefore, when the full kinetic profile of the drug under study has been established, measurement in this or another fluid with higher concentrations could replace that in plasma, because the relationship between both data sets has then become known. We, however, dealing with the unravelling of this relationship, had to accept the inconvenience of derivative formation for GC in order to utilize the advantage of a much more sensitive detection. The next part of this chapter will deal with descriptions of the extractive alkylation technique and the nitrogen detector and their applications in the determination of chlorthalidone and mefruside in body fluids.
By extractive alkylation is meant a combined use of ion-pair extraction and subsequent alkylation of the acid-anion. Much theoretical and experimental work on ion-pair extraction has been carried out since about 1960 by Schill and coworkers (see e.g., Schill et al., 1977). A very extensive compilation of the literature in this field can be found in a review by Jonkman (1975). During the past decade, extractive alkylation has become a well-known derivatization technique for gas chromatographic analysis of a variety of weakly acidic compounds (Brandström and Junggren, 1969; Degen et al., 1976; Degen and Schweizer, 1977; Ehrsson, 1971; Ehrsson and Tilly, 1973; Ehrsson, 1974; Ervik and Gustavii, 1974; Fagerlund et al., 1979; Fleuren and van Rossum, 1978; Fleuren et al., 1979b; Gyllenhaal and Ehrsson, 1975; Hartvig and Fagerlund, 1977; Hartvig et al., 1978; Lindström, 1974; Lindström et al., 1975; Vessman et al., 1977). In the extractive alkylation process, acids are extracted in the anion form together with a positively charged counterion from a water phase into an organic phase having a poorly solvating ability, so that the anions possess a high reactivity in nucleophilic displacement reactions (Brandström and Junggren, 1969). Frequently, benzene or dichloromethane containing an alkylkalide, e.g. methyl iodide, are used in these reactions with a quaternary ammonium compound, e.g. tetrabutylammonium hydroxide, providing the counterion. The rate of alkylation increases with increasing lipophilic character and concentration of the counterion (Ehrsson, 1971; Ervik and Gustavii, 1974; Fagerlund et al., 1979). It depends also on the concentration of the alkylating agent and on the reaction temperature, as was observed by Ervik and Gustavii (1974) in determining optimum reaction conditions for extractive methylation of chlorthalidone. The reaction can be divided into two steps:

\[ \text{Q}^{+}_\text{aq} + \text{X}^-_\text{aq} \rightleftharpoons \text{QX}_{\text{org}} \]  
(1)

\[ \text{QX}_{\text{org}} + \text{CH}_3\text{I}_{\text{org}} \rightleftharpoons \text{CH}_3\text{-X} + \text{QI}_{\text{org}} \]  
(2)

where \( \text{Q}^{+}_\text{aq} \) and \( \text{X}^-_\text{aq} \) denote the concentrations of the cation (tetrahexylammonium ion) and chlorthalidone in an anion form, respectively, in the aqueous phase, and \( \text{QX}_{\text{org}} \) and \( \text{CH}_3\text{I}_{\text{org}} \) the concentration of the ion-pair and methyl iodide, respectively, in the organic layer. Although extraction of the ion-pair is not very high under equilibrium conditions, i.e. the ratio \( \text{QX}_{\text{org}}/\text{X}^-_\text{aq} \) is only 2.6, the equilibrium of step (1) is displaced to the right when alkylation takes place. A high methylation rate was favoured by choosing high concentrations of methyl iodide (0.5 M in \( \text{CH}_2\text{Cl}_2 \)) and by working at a temperature of 50°C. Under these conditions the reaction was
complete after 15 minutes (Ervik and Gustavii, 1974). We verified that chlorthalidone had been converted to its tetramethyl derivative, and that no hydrolysis of the isoindolin ring had taken place (Chapter 3). Quantities up to ca. 20 μg of chlorthalidone can be permethylated, which is by far sufficient for the assay of concentrations occurring in biological fluids. Mefruside was found to be dimethylated more easily, the reaction being complete within 5 minutes at room temperature (Chapter 4). Extensive descriptions of the complete analytical procedure can be found in the Chapters 3 and 4.

Because we felt that some details would deserve additional explanation, we present now already an abbreviated version of the method for chlorthalidone (Fig. 2.4). According to the extraction profile shown in Fig. 2.1

**Figure 2.4**
Flow-chart for the determination of chlorthalidone in biological fluids, using extractive methylation and gas chromatography with nitrogen detection.

<table>
<thead>
<tr>
<th>Phase in which drug is present</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma (1-2 ml)</td>
<td>Add buffer of pH 7.4-pH 9.0 (0.5-2 ml)</td>
</tr>
<tr>
<td>urine (1 ml)</td>
<td>(in case of red blood cells: 600 mg NaCl + 100 mg NaF in addition)</td>
</tr>
<tr>
<td>red blood cells (0.1-0.2 ml)</td>
<td>Add internal standard (5 μg, 0.1 ml)</td>
</tr>
<tr>
<td></td>
<td>Extract twice during 20 min with 10 ml methylisobutylketone/ethanol (100:2 v/v)</td>
</tr>
<tr>
<td></td>
<td>Centrifuge briefly</td>
</tr>
<tr>
<td></td>
<td>Transfer organic layers into another tube</td>
</tr>
<tr>
<td>methylisobutylketone/ethanol</td>
<td>Re-extract once with 2 ml 0.1 M NaOH during 15 min</td>
</tr>
<tr>
<td></td>
<td>Transfer alkaline layer into another tube</td>
</tr>
<tr>
<td>0.1 M NaOH</td>
<td>Add 50 μl of 0.1 M tetrahexylammonium-hydroxide (THA)</td>
</tr>
<tr>
<td></td>
<td>Mix well</td>
</tr>
<tr>
<td></td>
<td>Add 5 ml of CH₃I in CH₂Cl₂ (0.75 M)</td>
</tr>
<tr>
<td></td>
<td>Shake vigorously for 20 min at 50°C</td>
</tr>
<tr>
<td></td>
<td>Centrifuge briefly</td>
</tr>
<tr>
<td></td>
<td>Transfer organic layer into another tube</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>Evaporate to dryness to remove CH₃I (40°C)</td>
</tr>
<tr>
<td></td>
<td>Add 5 ml of n-hexane</td>
</tr>
<tr>
<td></td>
<td>Sonificate during 5-10 min</td>
</tr>
<tr>
<td></td>
<td>Centrifuge to precipitate insoluble THA-salt</td>
</tr>
<tr>
<td></td>
<td>Transfer supernatant to another tube</td>
</tr>
<tr>
<td>n-hexane</td>
<td>Evaporate to dryness</td>
</tr>
<tr>
<td></td>
<td>Re-dissolve in 50 μl of ethanol</td>
</tr>
<tr>
<td>ethanol</td>
<td>Inject 5 μl into gas chromatograph with nitrogen detector</td>
</tr>
</tbody>
</table>
chlorthalidone can be extracted almost quantitatively from aqueous buffers until ca. pH 9 with methyl isobutyl ketone. This holds true also for extraction from plasma and urine, but not for that from red blood cells or whole blood, where extraction recovery is reduced due to strong binding of chlorthalidone to constituents in the erythrocytes, as will be pointed out in Chapter 3. In this case, an equal recovery as that from buffer alone was achieved by saturating the erythrocytes with a mixture of sodium chloride and sodium fluoride. Normally, we used an extraction pH of 7.4 for all plasma, red blood cell and urine samples, but incidentally a pH of 9 proved to be more advantageous, e.g. in case of bile assay (see also the Chapters 3, 11 and 13).

As indicated in Fig. 2.4 the 0.1 M tetrahexylammonium hydroxide solution should be added to the 0.1 M NaOH volume prior to addition of the dichloromethane layer. This at first sight unimportant precaution proved to avoid the occurrence of an enormous disturbing signal in the gas chromatogram, eluting immediately after the solvent peak. We ascribed this to decomposition of the quaternary ammonium salt to its amine, as this is known to occur in more concentrated solutions (Roberts and Caserio, 1965). Furthermore, we observed that the presence of chloride ions, e.g. 0.005 M in the aqueous alkaline layer negatively influenced the methylation of chlorthalidone, which may be attributed to competition for the positive counterion with respect to ion-pair extraction into the organic phase. This circumstance stressed the necessity of cleaning all glass tubes with distilled water prior to use and of avoiding contamination which could possibly occur by incautious transfer of the organic extracts of plasma and, especially, red blood cells to the next step of the procedure. Therefore, always brief centrifugation was carried out for good separation of these layers.

The dissolution step with n-hexane was employed to get rid of the tetrahexyl ammonium iodide, the by-product of the extractive methylation, see reaction step (2), which is almost not soluble in this solvent. Removal of this substance is necessary prior to injection of the methylated derivatives into the gas chromatograph, because of the enhanced sensitivity of the nitrogen detector for nitrogen-containing organic compounds (this step is, however, because of the iodide, also necessary in case of electron-capture detection, as reported by Ervik and Gustavii, 1974).

Finally, the residue was dissolved in ethyl alcohol, because n-hexane causes a much larger heat of combustion when introduced into the alkali flame ionization detector. As the operation of this detector is temperature-dependent, as outlined in the following section, an unwanted change in detector sensitivity would result then.

Despite the fact that the method consists of several steps, see Fig. 2.4, the overall procedure could be performed, by use of an appropriate inter-
Figure 2.5
Repeated assay of plasma samples indicating good reproducibility of the method of determination of chlorthalidone. The investigations, of which the results are shown in this plot, are described in Chapter 9.

nal standard, with a good reproducibility (5% standard deviation between concentrations of 10 ng/ml and 2.5 µg/ml, Chapter 3). To illustrate this, Fig. 2.5 shows the results of duplicate determinations on different days of a series of plasma samples, obtained from two typical experiments in man.

THE NITROGEN DETECTOR IN GAS CHROMATOGRAPHY

The most striking property of the nitrogen detector is its ability to selectively measure nitrogen over other elements in organic molecules (over carbon in even a 5000-fold ratio), independently of their further chemical class, the output signal being linearly proportional to the nitrogen content of the molecule (Burgett et al., 1977; Maier-Bode and Riedmann, 1975). This detector belongs to the group of the so-called thermionic detectors using an alkali metal salt, of which the phosphorus detector is another variant. The detection mechanism of a nitrogen (or phosphorus) detector does not involve measurement of the ionization current of the eluted substances, as with other ionization detectors (electron capture detector, flame ionization detector). Instead, changes in the degree of ionization of an alkali metal present in the detector room (e.g. rubidium bromide) are
monitored. The exact nature of the response is, however, not completely known. Several mechanisms have been proposed, and are reviewed by Sevcik (1976). In general, it is believed that the background current is a result of the ionization of the vaporized alkali atoms (Aue et al., 1967; Brazhnikov, 1970; Page and Woolley, 1968; Sevcik, 1976). This ionization, in turn, depends only upon the temperature of the surroundings. Neither hydrogen nor combustion products need to be present for this ionization to occur. Also the presence of the flame on itself is not crucial, because flameless nitrogen detectors have been demonstrated to give similarly selective responses (Burgett, 1977; Kolb and Aue, 1977). In these cases, ionization was achieved by using an electrically-heated device.

The reactions which occur when nitrogen compounds enter the detector chamber are not fully understood. Mostly, it has been suggested that the organic nitrogen-containing molecules are pyrolyzed to form cyano radicals, which remove an electron from the vaporized alkali (Aue et al., 1967; Kolb and Bischoff, 1974; Maier-Bode and Riedmann, 1975). These cyanide ions then combine at the collector to form hydrogen cyanide and thereby generate an increased output signal. The ionized alkali returns to the source surface to be re-evaporized. However, as discussed by Sevcik (1976), also other mechanisms have been proposed.

Regardless from the mechanism of response, the nitrogen detector has proven to be particularly well-suited for analysing low concentrations of nitrogen-containing drugs in a wide variety of chemical classes. So, determinations in biological fluids have been reported on e.g. barbiturates (Breimer and van Rossum, 1974; Goudie and Burnett, 1973; Riedmann, 1974), phenothiazines (Jonkman et al., 1975b; Riedmann, 1974), anti-epileptic drugs (Goudie and Burnett, 1973), antihistamines (Bilzer and Gundert-Rémy, 1973; Smith et al., 1978), tricyclic antidepressants (Bailey and Jatlow, 1976), 5-fluorouridine (De Leenheer and Gelijkens, 1978) and theophyllin (Lowry et al., 1977). This encouraged us to apply the nitrogen detector to determination of sulfonamide diuretics (Fleuren and van Rossum, 1978; Fleuren et al., 1979b; Chapters 3, 4 and 5).

The first device utilizing the thermionic principle in combination with gas chromatography was described by Karmen and Giuffrida (1964) for the detection of phosphorous and chlorinated hydrocarbons. Aue et al. (1967) published the first application of the alkali flame ionization detector towards nitrogen-containing compounds. The selectivity towards the different hetero-elements depends upon the nature of the alkali salt, rubidium bromide being favourable for detection of nitrogen (Aue et al., 1967; Brazhnikov, 1970).

The Hewlett-Packard nitrogen detector employed in our investigations is a modified flame ionization detector, operating also with rubidium bromide as the alkali source. A rubidium bromide crystal has been made
from the molten salt and has been given a cylindrical form with a central bore in its longitudinal axis. This crystal fits into the cylindrical collector electrode. The other electrode is mounted concentrically with the burner-jet top. The distance between the crystal and the flame can be adjusted mechanically. As pointed out above, the temperature of the crystal surface determines the evaporation rate of the alkali source and thereby the output signal and the selectivity. Therefore, all parameters causing a rise in temperature of the crystal are of influence. These include, besides the position of the crystal, the gas flow rates (hydrogen, oxygen and carrier gas), detector base temperature and internal diameter of the burner-jet. Proper adjustment is critical for obtaining maximal sensitivity and selectivity of the nitrogen detector. These conditions have very extensively been described by Maier-Bode and Riedmann (1975) for the detector type employed by us and are only briefly indicated, therefore, in the gas chromatographic sections of the Chapters 3 and 4.

**SUMMARY**

Principles of organic solvent extraction of sulfonamide diuretics in general and chlorthalidone and mefruside especially from biological fluids were discussed on basis of physico-chemical properties of these drugs, viz. lipophilicity and acid ionization constants.

The literature dealing with the analysis of diuretics, starting with the late fifties, when these drugs were introduced, until 1979 was reviewed. Early colorimetric assays based upon the Bratton-Marshall reaction appeared to lack not only specificity but also sensitivity. Current methods of determination, employing high-performance thin-layer chromatography, high-pressure liquid chromatography or gas chromatography equipped with selective detectors, were compared. It was concluded that, up to now, only gas chromatography with electron capture or nitrogen detection has enabled measurement of low nanogram per milliliter concentrations of sulfonamide diuretics in body fluids. For a few drugs showing favourable fluorescence properties thin-layer chromatography with fluorometric detection could also be employed. High-pressure liquid chromatography appeared to be very convenient for the assay of sulfonamide diuretics in urine, but the limit of detection of this technique did not suffice for studying plasma kinetics of these drugs.

After administration of single doses to humans, plasma concentrations of many diuretic drugs are in the very low nanogram per millimeter range, so that the most sensitive technique - gas chromatography with nitrogen or electron capture detection - is required for fundamental pharmacokinetic
investigations. Prior to gas chromatography, sulfonamide diuretics have to be converted to volatile derivatives. Complete derivatization could at best be achieved by means of extractive alkylation. Background and applications of this technique were described, focusing on the determination of the sulfonamide diuretics under study. The operation of the nitrogen detector, by which chlorthalidone and mefruside were determined down to 5 ng/ml in plasma, was outlined.

APPENDIX 2.1

The two proton dissociation equilibria of a dibasic acid in aqueous solution can be described by:

\[
K_{a1} = \frac{[H^+]_{aq} \cdot [A^-]_{aq}}{[HA]_{aq}} \quad \text{or} \quad \frac{[HA]_{aq}}{[HA]_{aq} + [A^-]_{aq}} = \frac{1}{pH - pK_{a1}}
\]

(Eq.2.1)

and

\[
K_{a2} = \frac{[H^+]_{aq} \cdot [A^{2-}]_{aq}}{[A^-]_{aq} \cdot [A^{2-}]_{aq}} \quad \text{or} \quad \frac{[A^-]_{aq}}{[A^-]_{aq} + [A^{2-}]_{aq}} = \frac{1}{pH - pK_{a2}}
\]

(Eq.2.2)

where \( pH = -\log[H^+]_{aq} \), \( pK_{a1} = -\log K_{a1} \), \( pK_{a2} = -\log K_{a2} \), and \( K_{a1} \) and \( K_{a2} \) are the first and second dissociation constants*, respectively. \([HA]_{aq} \), \([A^-]_{aq} \) and \([A^{2-}]_{aq} \) represent the concentrations of the unionized, single ionized and double ionized species of the weak acid, respectively.

*pK_{a1} and pK_{a2} denote in this appendix the apparent acid dissociation constants, which are smaller than the thermodynamic dissociation constants due to the ionic strength of the buffer solution used (cf. Chapter 6).
When a weakly acidic drug is partitioned between an aqueous and an organic phase, the relative amount present in the organic layer after equilibration will depend upon the extraction pH. For equal phase volumes and unsaturated solutions, the ratio of the concentration in the organic phase over that in the aqueous phase is called apparent partition coefficient (APC):

\[
\text{APC} = \frac{[\text{HA}]_{\text{org}} + [\text{A}^-]_{\text{org}} + [\text{A}^{2-}]_{\text{org}}}{[\text{HA}]_{\text{aq}} + [\text{A}^-]_{\text{aq}} + [\text{A}^{2-}]_{\text{aq}}} \quad (\text{Eq. 2.3})
\]

If we assume that only the unionized species enters the organic phase (which will be allowed in general, except e.g. in case of ion-pair formation), Eq. 2.3 is reduced to:

\[
\text{APC} = \frac{[\text{HA}]_{\text{org}}}{[\text{HA}]_{\text{aq}} + [\text{A}^-]_{\text{aq}} + [\text{A}^{2-}]_{\text{aq}}} \quad (\text{Eq. 2.4})
\]

The true partition coefficient (TPC) can be regarded as a measure for the intrinsic lipophilicity of a drug (apart from ionization):

\[
\text{TPC} = \frac{[\text{HA}]_{\text{org}}}{[\text{HA}]_{\text{aq}}} \quad (\text{Eq. 2.5})
\]

To find the relationship between APC and TPC, we first have to combine Eq. 2.1 and Eq. 2.2 to:

\[
\frac{[\text{HA}]_{\text{org}}}{[\text{HA}]_{\text{aq}} + [\text{A}^-]_{\text{aq}} + [\text{A}^{2-}]_{\text{aq}}} = \frac{1}{1 + ((1+10^{\text{pH-pKa}_2})\cdot10)} \cdot \frac{\text{pH-pKa}_2}{\text{pH-pKa}_1} \quad (\text{Eq. 2.6})
\]

Now, it can be readily derived from Eqs. 2.4, 2.5 and 2.6 that

\[
\text{APC} = \frac{\text{TPC}}{1 + ((1+10^{\text{pH-pKa}_2})\cdot10)} \cdot \frac{\text{pH-pKa}_2}{\text{pH-pKa}_1} \quad (\text{Eq. 2.7})
\]
The extraction ratio (E) can be defined as the ratio of the amount of drug in the organic phase over the sum of the amounts in aqueous and organic phases, at any pH:

\[
E = \frac{[\text{HA}]_{\text{org}} \cdot V_{\text{org}}}{([\text{HA}]_{\text{aq}} + [\text{A}^-]_{\text{aq}} + [\text{A}^{2-}]_{\text{aq}}) \cdot V_{\text{aq}} + [\text{HA}]_{\text{org}} \cdot V_{\text{org}} - \frac{\text{APC}}{V_{\text{aq}} / V_{\text{org}} + \text{APC}}}
\]

(Eq. 2.8)

By use of Eq. 2.7 we obtain

\[
E = \frac{\text{TPC}}{V_{\text{aq}} / V_{\text{org}} (1 + (1+10^{\text{pH-pKa}_2}) \cdot 10^{\text{pH-pKa}_1}) + \text{TPC}}
\]

(Eq. 2.9)

Equation 2.9 offers a useful means for finding the TPC, once the partitioning of a drug as a function of the pH of the aqueous phase has been measured. This gives more accurate results than the calculation of TPC by measuring at low pH (cf. Eq. 2.5) because the ratio of $[\text{HA}]_{\text{org}}/[\text{HA}]_{\text{aq}}$ is very sensitive to small errors in either the aqueous or organic phase. The distribution curves of Fig. 2.1 were fitted to Eq. 2.9 by use of a non-linear least squares regression analysis program(such as FARMFIT, see Chapter 1). At first, we attempted to fit the experimental data to a simpler form, which holds true as ionization of the second acid group should be negligible:

\[
E = \frac{\text{TPC}}{V_{\text{aq}} / V_{\text{org}} (1+10^{\text{pH-pKa}_1}) + \text{TPC}}
\]

(Eq. 2.10)

It appeared, however, that the second acid group of chlorthalidone (with pKa2) strongly influenced the shape of the distribution curves at a higher pH, so that the fits according to Eq. 2.9 were much better than those according to Eq. 2.10. This was judged by visual inspection, the sum of the squared deviations and the symmetry of residuals (cf. Chapter 6). Because
the pK\textsubscript{a2} was not as accurately known as the pK\textsubscript{a1}, see Chapter 6, several values of pK\textsubscript{a2} were tried until the lowest sum of squared deviations had been found (cf. Chapter 6). This occurred at pK\textsubscript{a2} = 10.95.

For a rough graphical estimation of the TPC, the following may be used. At the pH where half of the total amount of drug is extracted into the organic phase, so \( E = 0.5 \), we obtain (if pH\textsubscript{0.5} differs by at least one half log unit from pK\textsubscript{a2}):

\[
\text{pH}_{0.5} \approx \text{pK}_2 + \log \left( \frac{\text{TPC}_\text{org}}{\text{TPC}_\text{aq}} - 1 \right)
\]

REFERENCES


Rupp, W.A., Heidland, A., Neuhaus, G., Hajdu, P.: Pharmacokinetics of furosemide in sub-


CHAPTER 3

DETERMINATION OF CHLORTHALIDONE IN PLASMA, URINE AND RED BLOOD CELLS

INTRODUCTION

A sensitive and accurate assay in body fluids is necessary in order to determine the pharmacokinetic characteristics of a drug. Pulver et al. (1959) and Tweeddale and Ogilvie (1974) reported spectrophotometric methods for chlorthalidone, which, however, were not enough sensitive for the measurement of therapeutic plasma levels. The use of gas chromatography in the determination of chlorthalidone was delayed probably by the difficulty in forming a volatile derivative of this compound, which contains four polar groups (Fig. 3.1). Application of the extractive alkylation technique, of which a brief description has been given in Chapter 2, followed by gas chromatography with electron-capture detection, led to an analysis of chlorthalidone at the nanogram level, as described by Ervik and Gustavii in 1974. However, a complete analysis of a drug in body fluids must include the correct way of handling the biological samples, especially if variations herein influence the concentration values found. In this chapter it will become evident that the plasma concentration values of chlorthalidone are to a great extent dependent upon the method of separation of the plasma from the erythrocytes. Therefore, a proper treatment of the blood samples will be emphasized, besides the gas chromatographic part of the analysis. Use is made of the nitrogen sensitive mode of the flame ionization detector, which can measure nanogram concentrations of chlorthalidone, as was expected because the drug molecule contains two nitrogen atoms (see Chapter 2). An adequate internal standard (II in Fig. 3.1) is incorporated in the extraction procedure and special attention is paid to a complete recovery of the drug in the red blood cell assay.

Figure 3.1
Chemical structures of chlorthalidone (I) and the internal standard (II) used in the gas chromatographic analysis.

74
MATERIALS AND METHODS

Apparatus

Gas chromatographic analyses were performed on a Hewlett-Packard (HP, Avondale, Pa, USA) Model 5750 G gas chromatograph, equipped with a dual nitrogen detector (alkali flame ionization detector, A.F.I.D., with a rubidium bromide crystal; HP Model 15161 A) and connected to a HP recorder, Model 7127 A. Column: A glass column (1.8 m x 3 mm I.D.) packed with 3% GC-SE 30 on Gas Chrom Q, 100-120 mesh (Applied Science Labs., State College, Pa, USA) was used. Before being packed the glass column was cleaned with chromic acid, silanized with 5% dimethylidichlorosilane in toluene, rinsed with methanol and dried. The column was used as long as a good peak symmetry was present (usually 3-6 months). Prior to each analysis of a series of unknown samples, several pre-injections of the methylated derivative of chlorthalidone, e.g. obtained from assay on previous days, were performed to reduce adsorption to the column, as evidenced by tailing of peaks in the gas chromatogram. This proved to be especially important in the lower concentration range, from 10-50 ng/ml. Temperatures: Column 265°C (isothermal); Injection port 320°C. Detector 360°C. Gas flow-rates: The carrier gas was helium at a flow-rate of 60 ml/min. The hydrogen flow-rate was 30 ml/min and the air flow-rate 180 ml/min.

Operation of the nitrogen detector: the optimal use of an A.F.I.D. necessitates adjustment of constant gas flow-rates. The distance between the collector, containing the rubidium bromide crystal, and the flame is very important with respect to the sensitivity and selectivity for nitrogen-containing compounds. Also the crystal surface has to be clean and smooth. At the beginning of each day the crystal surface was wiped off with a soft brush and the collector was lowered until the maximum ionization current was reached (maximum recorder deflection, range 10^2, attenuation 32). The detector response was monitored by an injection of 5μl stock solution (500 ng chlorthalidone-tetramethylidervative), which gave full scale deflection at the recorder (range 10^3, attenuation 32).

A shaking apparatus (Marius, Utrecht, The Netherlands) was used for extractions and where shaking is indicated. Centrifugations were carried out in a Sorvall GLC2 centrifuge. For sonications a sonication bath (Branson 220, Branson, Soest, The Netherlands) was used.

Reagents

Chlorthalidone (1-oxo-3-(3'-sulfamoyl-4'-chloro-phenyl)-3-hydroxy-isindolin) was obtained as a gift from Ciba-Geigy, Basle, Switzerland. The internal standard, 4-chloro-N^1-methyl-N^1-(3-methoxypropyl)-l,3-benzene disulfonamide, was kindly supplied by Bayer, Wuppertal, G.F.R.
The purity of chlorthalidone and the internal standard was better than 99.5% as shown by thin-layer chromatography (silica gel 0.25 mm, on precoated glass plates with fluorescence indicator F254, Merck, Darmstadt, G.F.R.; solvent system: ethylacetate:n-hexane = 9:1 (v/v), RF chlorthalidone = 0.16; solvent system: methanol p.a., RF chlorthalidone = 0.72. The RF values of the internal standard are given in Chapter 4). Chlorthalidone stock solutions were prepared by dissolving 100 mg in 2000 ml Sörensen phosphate buffer pH 7.4 (0.067 M), and diluting to the appropriate concentrations. Internal standard solutions (50 and 5 μg/ml) were prepared in the same buffer. Ethanol, methyl isobutyl ketone, n-hexane and dichloromethane (all pro analysi grade; Merck, Darmstadt, G.F.R.) were used without purification. Methyliodide (Riedel de Haen, Hannover, G.F.R.) was used without purification. Tetrahexyl ammonium hydrogen sulphate was obtained from Labkemi, Stockholm, Sweden and converted to the hydroxide (0.1 M in aqueous solution) by dissolution in icecold 0.2 M sodium hydroxide. All inorganic chemicals used (E. Merck, Darmstadt, G.F.R.) were of pro analysi grade. Glassware was cleaned by brushing with detergent (Dubro, commercially available in The Netherlands), followed by thorough rinsing with tapwater, distilled water and ethanol. The heparin solution (Organon, Oss, The Netherlands) contained 50 mg (= 5000 U) per ml.

**Determination of chlorthalidone in plasma**

Into a glass-stoppered conical tube of 50 ml capacity were pipetted 1 ml of plasma and 1 ml of Sörensen phosphate buffer pH 7.4 (0.067 M) containing 5 μg internal standard, and the mixture was extracted twice (20 min, about 250 strokes/min) with 10 ml methyl isobutyl ketone-ethanol (100:2, v/v) in a shaking apparatus. From plasma samples expected to contain concentrations lower than about 25 ng/ml, also 2 ml portions, to which 0.5 ml of Sörensen phosphate buffer (0.5 M) and 0.1 ml of internal standard solution (50 μg/ml) had been added, were taken for analysis. After centrifugation for 5 min at 3000 r.p.m., the upper organic layers were transferred with a Pasteur pipet to another conical tube and extracted with 2 ml of 0.1 M NaOH (15 min, about 250 strokes/min). After centrifugation the aqueous layer was transferred to a 10 ml conical tube, equipped with a screw cap with a teflon-faced rubber liner. Next 50 μl 0.1 M tetrahexyl ammonium hydroxide solution was added and, after mixing, 5 ml of 0.75 M methyliodide in dichloromethane. The tube was shaken for 20 minutes at 50°C by means of a shaking apparatus (about 250 strokes/min) in a thermostated box. After centrifugation (5 min, 3000 r.p.m.) the organic layer was transferred to a tube (10 ml) with a finely tapered base and evaporated to dryness in a stream of purified dry air (water bath 40°C); 5 ml n-hexane was added and the tube was sonicated for 5 minutes in a sonication bath.
After centrifugation (5 min, 3000 r.p.m.) the hexane layer, containing the chlorthalidone-and internal standard-methylderivatives, was transferred to another 10 ml tube with a tapered base and the hexane was evaporated to dryness. To the residue 50 μl of ethanol were added, and about 5 μl were injected into the gas chromatograph by means of a Hamilton syringe. Each sample was injected twice, by using the dual column system of the gas chromatograph.

**Determination of chlorthalidone in erythrocytes**

The erythrocytes were whirl-mixed thoroughly and sonicated for 15 min, and a 200 μl volume was pipetted into a glass-stoppered tube of 50 ml capacity, containing 1.8 ml phosphate buffer of pH 7.4. Use was made of disposable Eppendorf pipettes; each pipet was washed with the buffer, so that no red blood cell material remained in it. Internal standard (0.1 ml, 5 μg) was added, and next 600 mg sodium chloride and 100 mg sodium fluoride, the tube contents were mixed and shaken for 15 minutes. Each sample was extracted and treated further exactly as described for the plasma samples.

**Determination of chlorthalidone in urine**

The volume and pH of each urine fraction were measured (pH meter 22, Radiometer, Copenhagen, Denmark) and the pH was adjusted to 8 by means of a few drops of 5.0 M NaOH. To 1 ml of urine in a conical tube were added 1 ml of 0.5 M Sörensen phosphate buffer of pH 7.4 and 0.1 ml internal standard solution (5 μg). The mixture was treated further as described for the determination in plasma.

**Preparation of calibration graphs**

The concentrations of chlorthalidone in plasma, red blood cells and urine were calculated by means of calibration graphs, obtained by adding known amounts of the drug to blank samples containing a constant amount of internal standard. In order to prepare the calibration graphs for the red blood cell assay, intact erythrocytes (0.2 ml) were incubated during 1.5 hours at 37°C in 1 ml phosphate buffer of pH 7.4 (0.067 M) containing known concentrations of chlorthalidone. This pre-incubation period was necessary to assure complete binding of chlorthalidone to red blood cells, as will be shown in the Results section below.

**Recovery studies**

The extraction yields of chlorthalidone from buffer and plasma into the organic layer were determined at three concentrations, 25 ng/ml, 250 ng/ml and 2.5 μg/ml, by adding known amounts to blank plasma and buffer of pH 7.4. After extraction, internal standard was added and the peak
area ratio after derivatization was calculated (ratio I) and compared with the value obtained after direct derivatization of chlorthalidone and internal standard (ratio II). Recovery was calculated as ratio I/ratio II times 100%. For the internal standard the extraction recovery was determined in the same way.

*Taking of blood samples*

Blood (sampling volume about 7 ml) was collected in heparinized glass tubes (containing one drop of heparin solution) and centrifuged (3000 r.p.m., 3 min) immediately after taking (within 5 sec.). Directly hereafter the plasma layer was removed rapidly with a Pasteur pipet, and was centrifuged additionally for 20 min (3000 r.p.m.). The plasma was decanted into another tube. Also the red blood cell layer was centrifuged again 20 minutes at 3000 r.p.m. The remaining supernatant was removed completely and discarded. The plasma and red blood cell fractions were frozen at -20°C until assay.

*In vitro incubations*

In vitro incubations of chlorthalidone were carried out by gently agitating freshly taken heparinized blood (hematocrit values 0.40-0.50) from human volunteers, obtained after overnight fasting, in a water bath of 37°C in an open air atmosphere. The compound was dissolved either in a small volume of 0.1 M NaOH containing 0.3% NaCl (0.1 ml for 5 ml of blood) and added directly to whole blood, or first dissolved in the plasma and subsequently added to the red blood cells.

*Influence of hemolysis on chlorthalidone plasma concentrations*

Human blood portions, 100 ml, were taken from healthy subjects after overnight fasting (n = 3, age 23-26 years) under conditions preventing hemolysis, viz., using a wide-bore needle and carefully heparinizing the blood, according to Hanks et al. (1960). The blood was freshly mixed (9:1 v/v) with chlorthalidone in 0.9% NaCl (50 μg/ml) and incubated for 90 min at 37°C. An aliquot of this mixture was taken apart before the incubation in order to obtain blank plasma. After 60 min of incubation, a 2 ml blood sample was taken and hemolysed by repeated freezing and thawing (3 cycles). The incubated blood was divided in separate portions and to each of them a small volume of hemolysate was added, ranging from 0.025 to 0.5 vol. %. After centrifugation the hemoglobin concentration in each plasma sample was measured relative to blank plasma by the cyanomethemoglobin method, see e.g. Dacie and Lewis (1975), with a small modification: instead of 0.02 ml of whole blood and 4 ml of reagent, 0.5 ml of plasma was used and added to 2 ml of reagent. The chlorthalidone plasma concentrations were determined in duplicate.
Identification of the methyl derivative of chlorthalidone

The methyl derivative of chlorthalidone was prepared in mg-scale as follows: 100 mg chlorthalidone was dissolved in 60 ml 0.15 M NaOH in a round-bottomed flask, 25 ml 0.5 M tetrahexyl ammonium hydroxide was added, and after mixing, 375 ml 4.4 M methyl iodide in dichloromethane was added and the mixture was allowed to reflux at 40°C, with vigorous stirring, during 1.5 hours. The organic layer was separated and evaporated to dryness with a Rotavapor apparatus. Due to the low solubility of the chlorthalidone derivative in hexane (15 mg/100 ml) the residue was extracted by sonication 7 times with 100 ml portions of n-hexane, after which gas chromatographic detection showed insignificant amounts of product in the hexane extract. After centrifugation (5 min, 3000 r.p.m.) in order to precipitate the tetrahexyl ammonium salt, the combined hexane extracts were evaporated to dryness. A control extraction verified that the hexane layer contained only trace amounts of the insoluble tetrahexyl ammonium salt. The product was recrystallized from very little methanol and dried over P2O5 in vacuo (white crystals, m.p. 127-128°C). The reaction yield was at least 95% as judged by weighing and by comparison of the gas chromatographic peak areas with those of the microgram scale reaction.

Mass spectrometry of the derivative formed was performed on a LKB 9000 mass spectrometer (LKB, Bromma, Sweden), with direct inlet, and after gas chromatography on a 3% SE-30 column. High resolution mass measurements were obtained by means of a VG Micromass 70-70F apparatus equipped with an on-line computer system (VG Data System 2040; VG, Altrincham, England).

Nuclear magnetic resonance spectra of the methyl derivative dissolved in d6-dmso (50 mg/ml) were obtained with a Varian 360-60-MC-NMR apparatus with tetramethylsilane as internal reference.

Infra red spectra (in KBr) were recorded by means of a Perkin-Elmer 257 grating spectrophotometer. Ultraviolet absorption was measured with a Cary 118 apparatus (Varian, Palo Alto, California, USA), the compound being dissolved in methanol (1 mg/100 ml).

RESULTS AND DISCUSSION

Gas chromatography

The alkali flame ionization detector was used successfully in the determination of chlorthalidone in body fluids, both in the nanogram and microgram range. Typical gas chromatograms are shown in Fig. 3.2. The chlorthalidone-tetramethyl derivative and the internal standard dimethyl derivative are well-separated under the conditions used with retention times
Figure 3.2
Gas chromatograms showing typical concentrations of chlorthalidone in human plasma and human urine. In the left part of the figure a plasma concentration of 24.8 ng/ml was measured 100 hours after an oral dose of chlorthalidone (100 mg) to a healthy volunteer, in the right part an urine sample was analysed and found to contain a concentration of 5.10 µg/ml. Use was made of the nitrogen mode of the flame ionization detector. Chl. = chlorthalidone, i.st. = internal standard (methyl derivatives).

of 3.2 and 2.1 min respectively. Blank plasma, red blood cell and urine samples from normal human subjects show no peaks at the retention times of chlorthalidone and internal standard and thereafter; therefore, large numbers of samples can be injected subsequently over a short period of time. The detector response is linear over a wide concentration range. This is shown in Fig. 3.3, where a calibration graph of chlorthalidone added to plasma was prepared at concentrations from 20 ng/ml to 1 µg/ml. The calibration graphs obtained (n = 10) were always linear and passed through the origin but varied in their slope about 10% between different determination days. Therefore, always two or three standard samples consisting of internal standard (5 µg) and chlorthalidone (amount depending upon the
concentration range of the biological samples, e.g. 25 ng, 100 ng and 500 ng for plasma concentrations) were included in each series to calibrate the graph. This variation was not due to irregular extraction and/or derivatization conditions as identical samples prepared in different runs, injected in the gas chromatograph in the same period of time gave always identical responses, and the same sample could vary from day to day in peak-area ratios. This was attributed to somewhat different responses of the detector for chlorthalidone and internal standard, depending upon minor changes in the analytical conditions in the alkali flame ionization detector between different determination days (e.g. gas flows, position of the crystal). A similar observation on the nitrogen detector has been reported by Bilzer and Gundert-Rémy (1973).

Determination of chlorthalidone in plasma

Plasma concentrations of chlorthalidone can accurately be measured down to 5-10 ng/ml. The detection limit is smaller by a factor 5. Standard deviations were 5% at concentrations of 1 μg/ml (n = 8) and 10 ng/ml (n = 8). The between-assay reproducibility (different determination days) was the same as the within-assay reproducibility. The extraction recovery

Figure 3.3A
Calibration graph for the gas chromatographic determination of chlorthalidone in 1 ml of plasma with concentrations ranging from 20-1000 ng/ml.

Figure 3.3B
Calibration graph for the gas chromatographic analysis of chlorthalidone in red blood cells, in the concentration range 0.25-25 μg/ml.
of chlorthalidone from plasma and phosphate buffer of pH 7.4 was 95% ± 2 (n = 4) at the concentrations investigated. The addition of 2 percent (v/v) of ethanol to methyl isobutyl ketone was found to increase the extraction recovery of the internal standard from 80% to 95%. The extractive alkylation reaction necessary for the methylation of chlorthalidone, in which the compound is extracted as an ion-pair with the tetrahexyl ammonium ion as the counterion and subsequently methylated with methyl iodide, as is outlined in Chapter 2, rapidly goes to completion under the experimental conditions (Ervik and Gustavii, 1974). By means of gas chromatography-mass spectrometry, it was confirmed that also the internal standard was completely di-methylated at the nitrogen atom of the free sulfonamide group by this procedure (Mass spectrometric data of the internal standard can be found in Chapter 5).

In a few occasions, plasma extracts were found to be contaminated by compounds of unknown origin giving rise to a broader "solvent peak" in the gas chromatogram than that usually observed. An excellent purification of these samples was achieved by an additional step in the isolation procedure prior to the methylation, viz. the washing of the 0.1 M NaOH layer with a 10 ml volume of methyl isobutyl ketone-ethanol (98:2 v/v). This procedure was also advantageous in the determination of very low plasma concentrations (below 25 ng/ml) by reducing the background of the gas chromatogram. Previously, it had been checked that the linearity of the calibration graph remained intact and that the recoveries of the drug and the internal standard did not diminish, when this purification step was applied.

Another possibility, which we employed incidentally (see e.g. Chapter 13), was extraction of plasma buffered at pH 9.0 (citrate-phosphate-borate buffer, 0.1 M, Teorell and Stenhagen) followed by two washes of the organic layer with 2 ml of the same buffer, after which the procedure was continued further as described in the experimental section of this chapter. This method gave the same quantitative results as those obtained by extraction at pH 7.4.

If required, additional purification of plasma extracts at a later stage of the procedure was achieved by washing the hexane layer twice with 2 ml of a saturated aqueous silver sulphate solution. The rationale behind this step may be that ion-pairs of tetrahexyl ammonium with endogenous substances in plasma (of unknown nature) are formed. Such nitrogen-containing compounds cause a tailing base-line in the gas chromatogram (unpublished observation). Tetra-alkyl ammonium sulphate ion-pairs are very insoluble in apolar organic solvents (Tilly, 1973), so that tetra-alkyl ammonium ions can be removed with a sulphate containing aqueous phase (Ehrsson, 1974).
Determination of chlorthalidone in urine

Preliminary experiments showed that the pH values of all urine samples to be extracted ranged between 7.40 and 7.45, if treated as described above. Calibration graphs were linear and the recovery and standard deviations were the same as those obtained in the plasma determination.

Determination of chlorthalidone in erythrocytes

Because of the strong binding of chlorthalidone to red blood cells (this thesis, Chapters 7 and 8), it was not possible to remove the drug quantitatively from this tissue by extraction with organic solvent alone. Extraction recoveries were 65% at a concentration of 10 μg/ml and only 50% at a concentration of 1 μg/ml in the red blood cells, if extracted twice with methyl isobutyl ketone-ethanol (98:2 v/v). Complete hemolysis of 0.2 ml red blood cells by freezing and thawing (10 times) in 2 ml of twice-distilled water recovered only 85% of the amount extracted from buffer pH 7.4 at a red blood cell concentration of 5 μg/ml. By exposing the red blood cells to salt saturation, by means of NaCl and NaF, a reliable method of determination of chlorthalidone in red blood cells was achieved. Calibration graphs were linear over the whole concentration range of 0-25 μg/ml, see e.g. Fig. 3.3, with standard deviations of 5% (n = 40). The extraction recovery was equal to that from plasma and urine in this procedure. At concentrations above 25 μg/ml, measurements were performed after reducing the volume of the erythrocytes to e.g. 0.1 ml. In calculating the red blood cell concentration of chlorthalidone, the values were corrected for a plasma inclusion of 6 volume percent. This correction factor applies to the amount of plasma trapped in the packed red blood cells at the relative centrifugal force and the duration of centrifugation used, see e.g. Chaplin and Mollison (1952). Degen and Schweizer (1977) reported that a maximum recovery of chlorthalidone from whole blood had been obtained by extraction with buffer of pH 5.5. We checked this for red blood cell samples. The concentrations of chlorthalidone, found by extracting 0.2 ml of well-homogenized human erythrocytes at pH 5.5 (mixed with 1.8 ml of 0.25 M citrate-phosphate-borate buffer, Teorell and Stenhagen), were identical with the values from the salt saturation method at pH 7.4, so that this extraction pH might be considered as an alternative for erythrocyte assay of chlorthalidone.

Stability of chlorthalidone in frozen plasma, urine and red blood cells

Upon storage at -20°C, the concentrations of chlorthalidone in plasma, urine and red blood cell samples from several human subjects had not changed after one year.
Taking of the blood samples and studies concerning the distribution of chlorthalidone between plasma and erythrocytes

During the investigations it became apparent that the transport process of chlorthalidone from plasma to erythrocytes was not complete in the first hours after ingestion of the drug. This resulted in great differences between concentrations of plasma samples, which were separated from the erythrocytes immediately after taking the blood, and plasma concentrations of the same blood samples, which had stood for a certain period of time. This is shown in Fig. 3.4, where plasma concentrations were determined after an oral dose of 100 mg Hygroton® (chlorthalidone) in two healthy human volunteers. The plasma concentrations measured after the

![Figure 3.4](image)

**Figure 3.4**

Lower curves: Plasma concentrations of chlorthalidone vs time on semi-logarithmic scale in two healthy volunteers after an oral dose of 100 mg Hygroton®. Vertical bars connect plasma concentrations determined in exactly the same blood samples, only differing in the time at which the blood was centrifuged after vein puncture. This figure illustrates the necessity of rapidly separating the plasma from the erythrocytes in the assay procedure of chlorthalidone in order to perform reliable plasma measurements especially during the first hours after absorption of the drug.

Upper curves: The red blood cell concentrations of chlorthalidone are 50-100 times higher than the corresponding plasma concentrations. Only the values obtained after direct centrifugation of the blood are shown.
standing of the blood were invariably lower than those after immediate centrifugation, while the greatest differences occurred in the first ten hours after administration*. This phenomenon was essentially the same with the other subjects studied in this respect (n = 4, not shown here).

The concentrations encountered in the red blood cells are about 50-100 fold higher than the plasma concentrations (see Fig. 3.4). Therefore, relatively large changes in plasma concentrations were not expected to influence the chlorthalidone concentration in the erythrocytes significantly, with the exception of the very early time points (e.g. low concentrations at time = 1 hr of subjects L.V. and V.F. in Fig. 3.4). This was confirmed experimentally (not shown here).

By in vitro incubations at 37°C it was shown that an immediate centrifugation of the blood samples minimized the exchange of chlorthalidone between plasma and red blood cells. Chlorthalidone was dissolved in plasma of fresh heparinized blood, at different concentrations. The plasma was mixed again with the red blood cell fraction and centrifuged either immediately (within 5 sec) or at the times 5, 10, 20, 30, 60, 150 min after mixing, during 3 minutes at 3000 r.p.m. From the time course of equilibration it became clear that the distribution equilibrium was reached after 60 min (Fig. 3.5). Furthermore, in the plasma samples centrifuged at t = 0 always the initial (added) plasma concentrations were measured. This was the case both in the situation where a high initial gradient of plasma vs. red blood cell concentration exists (Fig. 3.5A) as in the more in vivo like situation, where the erythrocytes contain already a certain amount of drug, and continuously more drug is added to the plasma from the gastro-intestinal tract. This in vivo effect was achieved by adding plasma to erythrocytes, which had been preincubated with chlorthalidone (Fig. 3.5B). The recovery at t = 0 (ratio measured concentration in plasma over added concentration in plasma) in experiments A and B was 100% and 99% respectively. From four of these experiments, we concluded that in blood samples centrifuged immediately after the vein puncture actually plasma concentrations were measured, which most closely resembled the in vivo concentrations of chlorthalidone. No change in concentration was observed when the for 3 minutes centrifuged plasma fraction was spun for an additional 20 minutes, after being removed from the red blood cells. This second centrifugation served to remove the buffy layer, so that clear samples were obtained.

* Also in the after-distribution period (24 hours and later) small but distinct differences between directly separated and after one hour separated plasma samples were found, even if the blood was kept at 37°C. This aspect may be of interest for the assay of plasma samples, for which centrifugation cannot be performed immediately, e.g. from patients outside the laboratory or clinic.
Chlorthalidone concentration in plasma and red blood cells (μg/ml)

16
14
12
10
8
6
4
2
0

0 30 60 90 120 150 Time (min)

A

- plasma
○ red blood cells

8
6
4
2
0

0 30 60 Time (min)

B

Figure 3.5
Time course of the in vitro transport of chlorthalidone from plasma to red blood cells in two incubations with whole human blood at 37°C. The equilibrium was complete after 60 minutes. The plasma concentrations were determined after immediate separation of the plasma from the red blood cells. In this way the measured plasma concentrations at t = 0 were equal to the added concentrations at t = 0 (complete recovery). For further explanation see text.

Influence of duration of centrifugation on chlorthalidone plasma concentrations.
In a separate experiment, 50 ml of blood was taken from a human subject 3 hours after a single oral 100 mg dose of chlorthalidone. Immediately after collection, blood was divided and centrifuged during either 1, 3, 5 or 10 minutes (by using four laboratory centrifuges simultaneously) and plasma was separated immediately thereafter. Duplicate analysis of plasma showed that the concentration in all four samples was the same. Especially the finding from the 10 minutes centrifugation period might appear surprising, because incubations at 37°C, see Fig. 3.5, had shown a rapid transport from plasma to erythrocytes during this time. The conditions of centrifugation differed, however, in two respects from those present during incubation. Firstly, erythrocytes were packed together during centrifugation, thereby strongly reducing the area of exchange with plasma. This seemed a plausible explanation, because plasma separated almost completely from red blood cells by a 3 min duration of centrifugation. Second-
ly, the transport rate of chlorthalidone to erythrocytes is much smaller at 20°C than at 37°C (see Chapter 8). (If centrifugation of blood is carried out at room temperature, a blood temperature close to the surrounding temperature is reached within a few minutes, as evident from our studies concerning mefruside, described in Chapter 4).

Concludingly, the exact duration of centrifugation, if started immediately after vein puncture, is not very critical within the limits described above, to obtain correct plasma concentrations of chlorthalidone. In all our investigations on chlorthalidone, reported in this thesis, we used a 3 min centrifugation period, because this was the shortest time to yield sufficient plasma for assay.

Influence of heparin on distribution between plasma and red blood cells

To study a possible influence of heparin on the distribution of chlorthalidone between plasma and erythrocytes, blood samples were drawn by vena puncture from 4 patients using Hygroton® and every blood sample was divided into two portions, one being placed in a heparinized tube and the other in a tube which contained no anticoagulant. The blood was centrifuged immediately after vein puncture. No differences between the plasma concentrations in heparinized tubes and those in tubes without anticoagulant were found. An effect of heparin was also not apparent after the in vitro incubations of chlorthalidone in whole blood, because of the consistency of the distribution ratio at different heparin concentrations, up to 10 fold the normal amount (0.2 ml of heparin solution on 100 ml of whole blood).

Influence of hemolysis on chlorthalidone plasma concentrations

Because the concentration of chlorthalidone in erythrocytes is 50-100 times higher than that in plasma, see Fig. 3.4, we expected that certain degrees of hemolysis would influence the plasma concentrations. A pilot experiment proved that the calibration graph of volume percentages of hemolysate added to blank plasma versus the spectrophotometrically determined hemoglobin (Hb) concentrations was linear and reproducible when using the blank plasma as the zero point. Thus, this method is adequate for measuring different degrees of hemolysis. However, it must be stated here that the Hb concentrations obtained are not absolute, as the assumption that the blank plasma contains no Hb at all was not strictly proved. Nevertheless, when blood was collected under conditions preventing hemolysis, very low hemoglobin concentrations in plasma were found, 0.16-0.58 mg/100 ml (Hanks et al., 1960), equivalent to 0.099-0.36 μmol/l. Hemolysis can be discerned with the naked eye if the plasma Hb concentration is above 6-12.5 μmol/l (De Gowin et al., 1949). This agrees with our observations that the plasma samples with the lowest degree of hemolysis
visible by the eye contained about 5 μmol/l of Hb in the Hb assay used. These considerations indicate that the Hb concentrations used (shown in Fig. 3.6) are not far from the absolute values (estimated maximally 5 μmol/l lower than the absolute values). When the chlorthalidone plasma concentration was plotted versus the plasma concentration of Hb a linear increase was found, with a constant slope (5.6 ng/10 μmol in Fig. 3.6; other experiments yielded comparable values). In Figure 3.6 the concentrations of chlorthalidone are given as percentages of the blank plasma value, which was 220 ng/ml for this subject. The slopes were in good agreement with the values expected theoretically on the basis of the whole blood concentration of chlorthalidone, the hematocrit values and the Hb concentrations of the blood portions used. On the basis of three of these experiments we decided to reject plasma samples with a Hb plasma concentration above 20 μmol/l, which corresponds to a hemolysis degree of ca. 0.12% (v/v, percentual volume of blood hemolysed). This degree of hemolysis could be very easily discerned with the naked eye as a substantial red colour; in cases of doubt, the Hb concentration was measured. In practice, when carrying out in vitro incubations of chlorthalidone in fresh blood taken the same day or a few days before, hemolysis seldom occurred. Mostly, the values for the Hb plasma concentrations after the incubation were maximally 5 μmol/l higher than before. Also in the 7 ml blood samples obtained
routinely for pharmacokinetic studies, hemolysis almost never amounted to a visible degree, although, of course, this depends upon the skill of the person performing the vein punctions.

**Equilibrium distribution between plasma and red blood cells in vivo**

The equilibrium distribution of chlorthalidone between plasma and erythrocytes in vivo was studied in a group of hypertensive patients using Hygroton® chronically (this thesis, Chapter 7). Blood samples were drawn by vena punction 24 hours after the last dose, in order to eliminate the problem of incomplete tissue distribution, which occurs in vivo during the first 10-15 hours after ingestion of the drug (see Fig. 3.4). Taking the blood samples shortly after the dose would have resulted in relatively high (non-equilibrium) plasma concentrations, see e.g. Figure 3.4.

**Identity of the chlorthalidone derivative formed in the extractive alkylation reaction**

In order to confirm the identity of the methyl derivative formed, electron impact mass spectra were recorded, and i.r., u.v. and n.m.r. measurements were performed. The mass spectrum shown in Fig. 3.7 was obtained after direct inlet into the ion source of the LKB 9000 mass spectrometer (temperature ion source 270°C, trap current 60 μA, voltage 20 eV), and indicates by the existence of the parent peaks m/e 394 (35Cl) and m/e 396 (37Cl) that a tetramethylderivative of chlorthalidone is present (chlorthalidone M.W. = 338.8). The fragmentation pattern after gas chromatography was identical. Furthermore, high resolution mass measurements of the peaks m/e 363, m/e 287 and m/e 176 were performed by means of a VG Micromass 70-70F mass spectrometer. The elemental compositions in agreement with these values were: for m/e 363: C17H16N2O3CIS, for m/e 287: C16H14NO2Cl, and for m/e 176: C10H10NO2. The u.v. absorption maximum was beyond 220 nm, and benzophenon absorption at and around 254 nm was absent. The i.r. spectrum showed one strong carbonyl absorption band at 1712 cm⁻¹ (5.83 μ). By comparison with chemical analogues of chlorthalidone (Graf et al., 1959) the combined u.v. and i.r. data could be attributed to the structure depicted in Fig. 3.7. The n.m.r. spectra yielded values of δ = 3.02 for the 6 methylprotons bound to the sulfonamide group, δ = 2.92 for the 3 protons of the methyl group bound to nitrogen in the isoindolin ring and δ = 3.16 for the 3 protons of the methoxy group; the 7 aromatic ring protons were between δ = 7.5 and δ = 8.2. The integrated values were in a ratio of 6:3:3:7, respectively. The mass spectrum shown in Fig. 3.7 was also given by the peak in the gas chromatogram with the retention time of derivatized chlorthalidone, present in the urine of a human subject following an oral dose of the drug. In addition, the retention time of the methylated chemical
hydrolysis product of chlorthalidone (G 32623: 4'-chloro-3'-sulfamoylbenzophenon-2-carboxylic acid, kindly provided by Ciba-Geigy) was found to be different from that of the original compound (2.8 min under the conditions of the GLC analysis). This product was not formed during the extractive alkylation of chlorthalidone. By this set of data it was considered sure that the substance determined in biological fluids is indeed intact chlorthalidone, not opened in the isoindolin ring by possible metabolic processes.

**SUMMARY**

A sensitive and specific gas chromatographic method is described for the quantitative determination of the diuretic and antihypertensive drug chlorthalidone (Hygroton®) in plasma, urine and red blood cells. After extractive methylation of chlorthalidone and the internal standard, the methyl
derivatives are measured by means of an alkali flame ionization detector (nitrogen detector). Amounts down to 10 ng in the biological sample can be measured accurately, with a standard deviation of 5%.

Because the concentration of chlorthalidone found in the erythrocytes is 50-100 times higher than that in the plasma, the influence of hemolysis on the plasma concentration has been investigated. In addition, a pharmacokinetic pilot study with human volunteers revealed that much too low plasma concentrations can be found (changes by more than 50%), if the plasma is not separated from the red blood cells immediately after the vein puncture. The emphasis is put on a correct handling of the blood samples, based upon in vitro incubations of chlorthalidone in human blood.

REFERENCES

INTRODUCTION

Mefruside (Fig. 4.1) is an effective diuretic agent (Horstmann et al., 1967; Meng and Kroneberg, 1967) and its biological fate has been investigated in rats and dogs (Duhm et al., 1967; Schlossmann, 1967). Very little information is available, however, about the disposition of this drug in man, as in a few instances only the sum of mefruside plus its metabolites was analysed by using either radioactivity measurements (Duhm et al., 1967) or spectrophotometry (Schlossmann, 1967). Both techniques have drawbacks with regard to their specificity and the latter also lacks sensitivity.

For the purpose of pharmacokinetic studies in man an accurate and sensitive method for the determination of mefruside in body fluids was required. In this study, the compound was made suitable for gas chromatography by extractive alkylation forming the dimethyl derivative and was detected by the use of an alkali flame ionization detector (nitrogen detector), which was expected to be advantageous because of its selectivity for organic nitrogen-containing compounds (Sevcik, 1976; Chapter 2). A structural analogue of mefruside was selected to serve as an internal standard (Fig. 4.1).

![Fig. 4.1. Structures of mefruside and internal standard.](image)
Because in preliminary human experiments much higher concentrations of mefruside in red blood cells than in plasma were found, in vitro incubations had to be performed to obtain information on the rate of distribution of the drug between the two blood components. Furthermore, drug partitioning was studied at different temperatures because the practical circumstances of vein puncture and centrifugation of the blood, routinely at room temperature, appeared to introduce a considerable change in the distribution equilibrium of mefruside between plasma and red blood cells. In this way the accuracy of the analytical procedure with regard to actual in vivo concentrations could be established.

MATERIALS

Reagents

Mefruside, 4-chloro-N\textsuperscript{1}-methyl-N\textsuperscript{1}-(tetrahydro)-2-methyl-2-furanyl)methyl-1,3-benzenedisulfonamide, and the internal standard, 4-chloro-N\textsuperscript{1}-methyl-N\textsuperscript{1}-(3-methoxypropyl)-1,3-benzenedisulfonamide, were gifts from Bayer (Wuppertal, G.F.R., courtesy of Dr. H. Horstmann). The purity of both compounds was larger than 99.5%, as evidenced by gas chromatography and by thin-layer chromatography (silicagel 0.25 mm, on pre-coated glass plates with fluorescence indicator F254, Merck, Darmstadt, G.F.R. Solvent system: toluene:ethanol:conc. acetic acid = 75:24:1 (v/v), \( R_F \) mefruside = 0.49, \( R_F \) internal standard = 0.45). Standard solutions of mefruside and the internal standard were prepared by dissolving 50.0 mg in 1000 ml of 0.067 M Sörensen phosphate buffer of pH 7.4. Dilutions of mefruside were prepared with the same buffer. Ethanol, n-hexane and dichloromethane (all pro analysis grade, Merck, Darmstadt, G.F.R.) were used without further purification. Iodomethane (Riedel de Haan, Hannover, G.F.R.) was also used without purification. The diethyl ether was either freshly destilled prior to the extractions or used as delivered by the manufacturer (containing 7 \( \mu \)g/ml of 2,6-di-tert.butyl-4-methylphenol as stabilizing agent, Merck, pro analysis grade), because identical results with the derivatization of the drug substances and no interference with the gas chromatographic detection were obtained for both solvent specifications. Tetrahexyl ammonium hydrogen sulphate (Labkemi, Stockholm, Sweden) was dissolved (0.1 M) in ice-cold 0.2 M aqueous sodium hydroxide. All inorganic chemicals used (Merck) were of pro analysis grade. The heparin solution (Thromboliquine) contained 50 mg (5000 U) per ml (Organon, Oss, The Netherlands). Glassware was cleaned by hand with a commercial detergent (Dubro), followed by thorough rinsing with tap water, distilled water and ethanol.
Gas chromatography

A Hewlett Packard (HP), model 5750 G, gas chromatograph with a dual nitrogen detector (alkali flame ionization detector), containing a rubidium bromide crystal (HP model 15161 A), was used in connection with a HP recorder, model 7127 A (HP, Avondale, Pa., U.S.A.).

Column: glass, 1.8 m long, 3 mm internal diameter, packed with 3% of SE 30 on Gas Chrom Q, 100-120 mesh (Applied Science Labs., State College, Pa., U.S.A.).

Temperature: oven 265°C (isothermal), injection port 320°C, detector 370°C. Gas flow rates: helium (carrier gas) 55 ml/min, hydrogen (detector gas) 30 ml/min, air (detector gas) 180 ml/min.

Before being packed, the glass column was treated with chromic acid, silanized with 5% dichlorodimethylsilane solution in toluene, rinsed with methanol and dried. In order to reduce adsorption to the packed column, especially at concentrations below 25 ng/ml, several pre-injections (5-10 times) of samples containing the methyl derivatives of mefruside and internal standard were necessary before each series.

For an optimal performance of the nitrogen detector, the gas flow rates have to be kept constant. The distance between the collector, with the crystal, and the flame is crucial to obtain maximal sensitivity and selectivity for nitrogen-containing molecules. Also, the crystal surface must be clean and smooth. Therefore, the crystal was wiped, at the beginning of each day, with a soft brush to remove silicon dioxide deposits and the collector was lowered until maximum ionization current (maximum recorder signal) was obtained (range 10²; attenuation 32). The detector response was monitored by an injection of ca. 500 ng of the dimethyl derivative of mefruside, which gave full-scale deflection of the recorder (range 10³; attenuation 32).

Other apparatus

A platform shaking apparatus (Marius, Utrecht, The Netherlands) was used for extraction and where shaking is indicated. Centrifugation was carried out in a Sorvall GLC 2 centrifuge (Meyvis, Bergen op Zoom, The Netherlands). For sonication a Bransonics 22 sonication bath (Branson, Soest, The Netherlands) was used. The pH was measured with a pH meter 22 (Radiometer, Copenhagen, Denmark). Haematocrit values were determined with a Hawskley micro-haematocrit centrifuge (Hawskley, Lancing (Surrey), England) and, after reading, corrected for a plasma trapping percentage of 3%, which applies to this method (England et al., 1972).
METHODS

Sampling of blood and urine

Blood samples, ca. 7 ml, were collected in heparinized glass tubes (containing one drop of heparin solution) and centrifuged immediately after taking (within a few seconds) for 3 minutes at 3000 r.p.m. Directly hereafter, the plasma layer was removed with a Pasteur pipet and transferred to another tube. The red blood cell layer was centrifuged for another 20 minutes at 3000 r.p.m. to obtain constant package of the erythrocytes (trapped plasma volume 6 vol. %, Chaplin and Mollison, 1952). The remaining supernatant was removed completely and discarded. The plasma layer was also spun during the same time, for practical reasons, in order to precipitate the buffy coat and so obtain clear samples, which were decanted into new tubes. The above treatment of the blood samples took place at room temperature (20°C) normally, with exception of parts of the partitioning experiments, described below. Whole blood samples, or plasma and red blood cell fractions, were frozen at -20°C until assay. Prior to analysis the samples were well homogenized by means of a whirl-mixer.

Blood for in vitro studies was collected in ca. 60 ml portions from healthy male volunteers (age 22-25 yr) after overnight fasting, under conditions preventing haemolysis, by using wide-bore needles and by carefully heparinizing the blood (0.1 ml heparin solution on 100 ml whole blood). Each portion was incubated with mefruside freshly, the same day, in an open air atmosphere and visible haemolysis did not occur. After collecting human urine, the volume and pH of each fraction were measured the same day and an aliquot of each portion was frozen at -20°C. Prior to assay the pH of the urine samples was adjusted to pH 8 with a few drops of 5 M sodium hydroxide solution.

Selection of derivatization time and temperature

The concentrations of the reactants of the extractive alkylation were chosen analogously to those described in Chapter 3 on the permethylation of chlorthalidone. Mefruside was dissolved (250 ng/ml) in 0.1 M NaOH and 2 ml portions of this solution were pipetted into glass tubes with a finely tapered base containing 50 μl of 0.1 M tetrahexyl ammonium hydroxide solution. After mixing, 5 ml of 0.75 M iodomethane in dichloromethane were added and the tubes were vigorously shaken at room temperature or at 50°C during either 1, 5, 10, 20 or 30 minutes. After brief centrifugation each organic phase was transferred to another tube containing 5 μg of the dimethyl derivative of the internal standard. The latter had been prepared by extractive alkylation at 50°C for 20 min, as a previous study had shown that complete derivatization had occurred under these conditions (Chapter 3). After evaporation of the combined substances to dryness (40°C), the
samples were processed in the same way as described after the evaporation step of the analytical procedure following.

**Analytical procedure**

Depending upon the kind of biological sample, one of the four following combinations was pipetted into a glass-stoppered conical 50 ml tube:

1) 2 ml of plasma and 0.5 ml of 0.5 M Sörensen phosphate buffer of pH 7.4
2) 0.5 ml of red blood cells and 1.5 ml of 0.067 M Sörensen phosphate buffer of pH 7.4
3) 1 ml of whole blood and 1 ml of 0.067 M Sörensen phosphate buffer of pH 7.4
4) 1 ml of urine and 1 ml of 0.5 M Sörensen phosphate buffer (pH 7.4)

Internal standard was added (5 μg, 0.1 ml) and the mixture was extracted twice (5 min, ca. 250 strokes/min) with 10 ml of diethylether in the shaking apparatus. After centrifugation for 5 min at 3000 r.p.m. the organic layers were transferred with a Pasteur pipet to another conical tube and the drug was extracted into 2 ml of 0.1 M of sodium hydroxide (10 min, ca. 250 strokes/min). After centrifugation (5 min, 3000 r.p.m.) the aqueous layer was transferred to a 10 ml conical tube, equipped with a teflon-faced rubber liner. Next, 50 μl of 0.1 M tetrahexyl ammonium hydroxide solution was added and, after mixing, 5 ml of 0.75 M iodomethane in dichloromethane. The tube was shaken for 15 min at 20°C by means of the shaking apparatus at ca. 250 strokes/min. After centrifugation (5 min, 3000 r.p.m.) the organic layer was transferred to a 10 ml tube with a finely tapered bottom and evaporated to dryness in a stream of purified dry air (waterbath 40°C); 5 ml of n-hexane was added and the tube was sonicated for 10 min. After centrifugation (5 min, 3000 r.p.m.) the hexane layer, containing the methyl derivatives of mefruside and internal standard, was transferred to another tapered 10 ml tube and the hexane was evaporated to dryness. To the residue 50 μl of ethanol was added and, after thorough whirl-mixing, 5 μl of the solution was injected into the gas chromatograph by means of a Hamilton syringe. Each sample was injected twice. The concentrations in the biological samples were calculated by the use of calibration graphs, obtained by plotting the mean ratio peak height mefruside/peak height internal standard against known amounts of mefruside added to blank samples with a constant amount of the internal standard.

**DRUG PARTITIONING BETWEEN PLASMA AND RED BLOOD CELLS**

**Rate of distribution in whole blood at 20 and 37°C**

Fresh human blood (60 ml portions) was centrifuged at 3000 r.p.m. for
10 min. Known amounts of mefruside, 50-100 μg, were dissolved in approximately one half of the total volume of plasma and preincubated for 30 min, either at 20 or at 37°C. The remainder of the blood portion was homogenized again and also kept at the desired temperature by gently agitating the flask in open air. After 30 min the blood was reconstituted, and separation of plasma and red blood cells was achieved by centrifuging (3000 r.p.m., 3 min.) 7 ml aliquots of the incubated blood at the following times: t = 0 (immediately after mixing), t = 1, t = 2.5, t = 5, t = 15 min. Plasma and whole blood concentrations were assayed according to the method described in the previous section. The plasma concentration, theoretically present at t = 0 before mixing of the blood constituents, was calculated from the amount of mefruside added and the total volume of the plasma, present in the incubation mixture, which was accurately known from the total volume of the blood portion and the corrected haematocrit value. Prior to, and during the incubations at 37°C, all apparatus and glassware were equilibrated at this temperature in a thermostatically controlled room.

Influence of temperature upon the distribution equilibrium

3 ml of mefruside solution (50 μg/ml in physiological saline) was added to 80 ml of fresh human blood and the mixture was divided into four portions, being placed at four different temperatures between 20°C and 37°C (actual values in Fig. 4.7). After gentle agitation for 10 min, duplicate 7 ml aliquots of each portion were centrifuged (3 min, 3000 r.p.m.) and the plasma layers were quickly removed, the whole procedure being carried out at thermostatically maintained temperatures. The temperature of each red blood cell fraction was checked after the centrifugation step.

The following experiment simulated the effect of handling blood samples at room temperature (20°C) upon the distribution of mefruside between plasma and erythrocytes, originally present at in vivo temperature (37°C): 20 ml human blood portions were spiked with mefruside at varying concentrations, ranging from 0.25-2.0 μg/ml (exact values in Fig. 4.8), warmed up and incubated for 5-10 min at 37°C in a waterbath kept in a thermostatically controlled box. Aliquots of each portion (7 ml) were centrifuged directly (3000 r.p.m., 3 min) by means of glass tubes and centrifuges conditioned at 37°C. Parallel to them, 7 ml aliquots were treated at room temperature (20°C) after the incubation. They were first drawn into a plastic syringe, transferred immediately thereafter to glass tubes and centrifuged (3000 r.p.m., 3 min), the plasma layers being rapidly removed with a Pasteur pipet and put into other tubes.
Gas chromatography and specificity

Figure 4.2 shows a typical gas chromatogram resulting from the assay of a human plasma sample, spiked with mefruside at a concentration of 12.5 ng/ml. The retention times of the internal standard and mefruside peaks are, respectively, 2.8 and 4.1 minutes and no interfering peaks are present at their positions in normal blank plasma, red blood cells, urine or whole blood. The only chemical species logically interfering with this assay could be a potential metabolite (M) of mefruside, liberated, if existing, by demethylation of the methyl-substituted sulfonamide group. By using propylidide (in analogy to the analytical procedure described for
methyl iodide) the propylated derivatives of mefruside and M were separated on the g.c. column, with retention times of resp. 5.1 and 6.1 min (conditions as described under Materials). No trace of M was detected (<1 ng/ml) in plasma, urine or whole blood of human subjects receiving single or repetitive doses of mefruside, in spite of the presence of considerable mefruside concentrations in the plasma (19-91 ng/ml) and red blood cells (0.61-2.64 µg/ml) of the patients under chronic treatment (n = 17). See Fig. 4.3. Therefore, the assay must be considered specific for the unchanged drug.

**Derivatization yield**
Mefruside is rapidly methylated at room temperature, the reaction being already complete after 5 minutes (Fig. 4.4). The structure of the methyl derivative was confirmed by mass spectrometry (20 eV, LKB 9000 mass
spectrometer), the most predominant mass fragment (m/e 85) was attributable to the 2-methyltetrahydrofuran moiety. A complete mass spectrum of dimethylated mefruside can be found in Chapter 5, where it was of help in the identification of 5-oxo-mefruside as a human metabolite. Also the internal standard methyl derivative was formed with a similar rate at room temperature, as was established in analogy to the experiment described for mefruside. Yet, in the analytical procedure a longer reaction time of 15 min was preferred, because it appeared that a strongly tailing substance, present in the methylation mixture, which eluted in the gas chromatogram just after the solvent peak, became reduced with longer reaction times, thus providing a more stable base-line, which was needed in the analysis of low concentrations of mefruside, below 25 ng/ml. Although the identity of this substance was not extensively studied, our observation that it disappeared completely from the gas chromatogram by washing the hexane layer with saturated aqueous silver sulphate solution (twice with 2 ml) points to a tetrahexyl ammonium salt. It is known that such a compound can be easily removed in this way (Ehrsson, 1974), due to the low organic phase partition coefficient of tetra-alkyl ammonium sulphate ion-pairs (Tilly, 1973).

**Extraction recovery**

The peak height ratio of mefruside and internal standard after extraction of mefruside from plasma or buffer of pH 7.4, followed by addition of the
Improvement in extraction recovery of mefruside from 2 ml of plasma (\(\Delta\), \(\cdot\)) and buffer (\(\triangle\), \(\circ\)) by two extractions with diethylether (10 ml) instead of a single one.

internal standard prior to the methylation (ratio I), was compared with the ratio obtained after direct derivatization of both compounds (ratio II). The extraction recovery was calculated as 100% times ratio I/ratio II. After one extraction with diethylether a value of 98% was found at a plasma concentration of 250 ng/ml, but the recovery decreased at lower concentration to ca. 80% at the 25 ng/ml level, see Figure 4.5. As this behaviour would not provide linear calibration graphs, the effect of an extra extraction was studied. Figure 4.5 shows that after two extractions a recovery of 99.5 \(\pm\) 0.5% (mean \(\pm\) S.D., \(n = 8\)) was obtained, independently of concentration, without differences in extraction yield from buffered plasma or buffer alone. Concludingly, solutions of mefruside in buffer can be used for the preparation of the calibration graph within a series of plasma samples. Analogously, it was established that the recovery from red blood cells (0.5 ml on 1.5 ml buffer) was the same as that from the buffer solution alone (see e.g. Figure 4.6).

**Calibration graphs and reproducibility**

Linear calibration graphs, passing through the origin, were obtained for both the determination in plasma and that in urine, red blood cells or whole blood. Some typical results are shown in Fig. 4.6. Between different days, variations in slope of about 15% occurred, due to differences in response of the detector to mefruside and internal standard, dependent
Fig. 4.6.
Typical calibration graphs for the gas chromatographic determination of mefruside in plasma (left part) and in red blood cells (right part).

upon minor changes in detector operation (e.g. gas flows, height of crystal) at different occasions. Variations in the response of this detector have been observed also by others (Gough and Sugden, 1973). No fluctuations of extraction and/or derivatization yields were evident as duplicate samples prepared in different series, but injected on the same day, always gave identical peak height ratios. Nevertheless, to check the whole procedure, two or three reference samples with amounts of mefruside in the expected range, e.g. 10, 50 and 150 ng/ml for plasma concentrations, were extracted, derivatized, and injected together with each series of unknown samples.

The reproducibility of the analysis was relatively constant over the whole concentration range, with coefficients of variation (% S.D.) of 3.6% (n=10) at 250 ng/ml, and 4.4% (n=8) at 10 ng/ml plasma.

In vitro uptake rate of mefruside by red blood cells

A very rapid transport of the drug from plasma to red blood cells was evident at both 37°C and 20°C. Table 4.1 shows that no systematic differences in the plasma concentrations were found within a 15 min period, starting from the very earliest time point (incubation time < 5 sec), the average ratio of red blood cell and plasma concentrations being $43.9 \pm 1.6$ (Table 4.1, left part). The exact transport rate was not measurable, due to the relatively long time-lag caused by the centrifugation of the blood (3
The same picture was obtained when erythrocytes were used which contained already a certain concentration of mefruside, thus simulating the in vivo situation, when during absorption of the drug from the gastrointestinal tract continuously more mefruside is added to the plasma and bound subsequently by the red blood cells: after adding a small amount of mefruside to the plasma of the preincubated blood portion (the one from the left part of Table 4.1) the drug was apparently bound with an equal rate, resulting in the same distribution ratio (right part of Table 4.1). Furthermore, a complete recovery of mefruside from whole blood can be seen by comparison of the added concentrations (at t = 0) with the analysed values. At 20°C very similar results (not shown) with respect to the rate of distribution were observed. Analogous experiments (not shown) revealed that mefruside was also released from the erythrocytes with a comparably rapid rate.

**TABLE 4.1**

Rate of distribution of mefruside in human whole blood at 37 °C at two different initial mefruside concentrations in the red blood cells: 0.00 μg/ml (left part) and 1.34 μg/ml (right part).

<table>
<thead>
<tr>
<th>incubation time (min)</th>
<th>C_{plasma} (μg/ml)</th>
<th>C_{whole blood} (μg/ml)</th>
<th>C_{red blood cells}/C_{plasma}</th>
<th>incubation time (min)</th>
<th>C_{plasma} (μg/ml)</th>
<th>C_{whole blood} (μg/ml)</th>
<th>C_{red blood cells}/C_{plasma}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0^b</td>
<td>1.13</td>
<td>0.615</td>
<td>0.00</td>
<td>0^b</td>
<td>0.0958</td>
<td>0.702</td>
<td>14.0</td>
</tr>
<tr>
<td>&quot;0&quot;^c</td>
<td>0.0302</td>
<td>44.4</td>
<td>1.5</td>
<td>&quot;0&quot;^c</td>
<td>0.0317</td>
<td>43.7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0307</td>
<td>43.7</td>
<td>2.5</td>
<td>1.5</td>
<td>0.0319</td>
<td>43.3</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.0310</td>
<td>43.3</td>
<td>5</td>
<td>2.5</td>
<td>0.0325</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.0320</td>
<td>41.9</td>
<td>15</td>
<td>5</td>
<td>0.0301</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.0292</td>
<td>46.3</td>
<td>15</td>
<td></td>
<td>0.0313</td>
<td>44.2</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD  
"0"-15 min ± 0.0306 ± 0.022 ± 1.3  
(n = 5)  

<table>
<thead>
<tr>
<th>incubation time (min)</th>
<th>C_{plasma} (μg/ml)</th>
<th>C_{whole blood} (μg/ml)</th>
<th>C_{red blood cells}/C_{plasma}</th>
<th>incubation time (min)</th>
<th>C_{plasma} (μg/ml)</th>
<th>C_{whole blood} (μg/ml)</th>
<th>C_{red blood cells}/C_{plasma}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0^b</td>
<td>0.0315</td>
<td>0.691</td>
<td>14.0</td>
<td>0^b</td>
<td>0.0315</td>
<td>0.691</td>
<td>14.0</td>
</tr>
</tbody>
</table>
| "0"-15 min ± 0.0009 ± 0.022 ± 1.3  
(n = 5)  

- Haematocrit (corrected) 0.451 ± 0.001 (mean ± SD, n = 10), left part and 0.488 ± 0.003 (mean ± SD, n = 10), right part.
- before mixing the blood constituents
- centrifugation started immediately after mixing (< 5 sec)
Effect of temperature change upon the distribution ratio of plasma and red blood cell concentrations

When the temperature of blood portions was lowered from 37°C to 20°C a considerable decrease (together ca. 35%) of the plasma concentrations of mefruside was observed, see Fig. 4.7, whereas the total blood concentration remained unchanged, thus indicating a change of the distribution ratio. This temperature-dependence of the distribution ratio and the very rapid rate at which the equilibrium between plasma and red blood cell concentrations was reached (previous section) necessitated a study of the quantitative changes of the plasma concentrations existing at in vivo temperature, caused by the decrease in temperature after treatment of blood samples (vein puncture, centrifugation) at routine circumstances (20°C). A pilot experiment had shown that after drawing 7 ml blood portions of 37°C into a 10 ml syringe, kept at room temperature, the temperature had fallen already to 31.7 ± 0.3°C (mean ± S.D., n = 5). After subsequent centrifugation for 3 minutes, the temperature of the samples was 27.5 ± 0.4°C (mean ± S.D., n = 5). Figure 4.8 presents a comparison of the plasma concentrations found after such routine treatment of the blood samples with those present if the entire procedure was carried out at 37°C. A linear distribution plot is apparent at both temperatures, implying a constant percentual difference between the actual (in vivo) concentrations and the experimentally determined values, which were on an average 85.4 ± 2.7% of the first ones (mean ± S.D., blood portions of two human subjects).

Fig. 4.7.
Temperature-dependent partitioning of mefruside between plasma and erythrocytes, evidenced by a decrease of plasma concentrations when going from 37 °C to 20 °C, while the total blood concentration remained constant (1.8 μg/ml).
Standing of blood samples after vein puncture during a certain period, e.g. 1 hr, results in a further lowering of the plasma concentrations, which was shown by in vivo experiments, in which plasma concentrations were determined after oral dosage of mefruside (see Fig. 4.9). Several of such studies (n = 4) revealed that the decrease after standing was much less regular than that after direct centrifugation of the blood. Therefore, a centrifugation immediately after vein puncture seemed by far preferable for pharmacokinetic studies, realizing that plasma concentrations obtained in that way are constantly ca. 15% lower than those present at 37°C, such that this treatment does not influence the assessment of plasma half-lives. Furthermore, it should be emphasized that plasma concentration decreases of this magnitude will barely influence the concentrations in the red blood cells if these are, which is the case (see e.g. Figure 4.10), about 30 times higher (an increase of only 0.5% was computed for blood of normal haematocrit). Therefore, plasma concentrations are a much more sensitive indicator for changes in the distribution ratio of this drug.
Fig. 4.9.
Plasma concentrations of mefruside after a single oral 50 mg dose to subject H.B. Exactly the same samples were used for both curves, the only difference consisted of the duration of the period between vein puncture and centrifugation. For further explanation see text.

APPLICATION

The present method was used to determine plasma and red blood cell concentrations of mefruside in man after administration of oral doses, as reported in Chapter 14. An example of a concentration-time curve after intake of a single oral dose is given in Figure 4.10. The concentrations in the red blood cells are about 30 times higher than those found in the plasma (not corrected for the constant percentual change caused by the fall of blood temperature, see Fig. 4.8). The time courses of the concentrations in
Plasma concentration and erythrocyte concentration curves on semi-logarithmic scale following an oral dose of 50 mg of mefruside to a human subject. After a rapid absorption, a biphasic decay can be seen without significant difference between the terminal half-lives in the two blood components. The concentrations in the red blood cells are about 30 times higher than those in plasma.

both blood components are exactly parallel to each other, with concentration maxima occurring at 2 hours after the dose. This constant ratio of plasma and erythrocyte concentrations is in agreement with the linear distribution pattern observed in vitro (Fig. 4.8) and with the rapid rate of equilibration (Table 4.1).
A specific gas chromatographic method for the determination of the unchanged diuretic drug mefruside in plasma, red blood cells or whole blood, and urine has been developed. Mefruside and an analogous internal standard are dimethylated by extractive alkylation and measured on a gas chromatograph equipped with a nitrogen detector (alkali flame ionization detector). Concentrations down to 5 ng/ml in plasma, using 2 ml samples, can be assayed accurately, with variation coefficients of 4.4% at the 10 ng/ml level and 3.6% at 250 ng/ml.

A very rapid partitioning of mefruside between plasma and erythrocytes was observed upon in vitro incubations in whole blood. Linear distribution plots, with concentrations in erythrocytes ca. 30 times higher than those in plasma, were found but their slopes were strongly temperature-dependent, such that at 20°C ca. 35% lower plasma concentrations than at 37°C resulted. However, if the plasma was rapidly separated from the red blood cells at room temperature, immediately after vein puncture, a reproducible and constant percentual decrease, ca. 15% different from the in vivo plasma concentrations, was obtained, thus making the procedure suitable for pharmacokinetic studies.

REFERENCES

Chaplin, H , Mollison, P L  Correction for plasma trapped in the red cell column of the hematocrit Blood 7, 1227-1238 (1952)
Horstmann, H , Wollweber, H , Meng, K  Chemische Struktur und diuretische Wirkung in der Reihe der 4-chlor-3-sulphonamido-benzolsulfonyl Arzneim -Forsch (Drug Res ) 17, 653-659 (1967)
Putter, J , Schlossmann, K  The degradation of mefruside. The participation of a "lactonase" in drug metabolism Biochim Biophys Acta 286, 186-188 (1972)


INTRODUCTION

In Chapter 4, we described a specific gas chromatographic method for determining mefruside in human body fluids (Fleuren et al., 1979). Because experiments with mefruside in man showed that less than 1% of the dose was excreted into urine as the unchanged drug (Chapter 14), it seemed logical to continue our investigations with a search for metabolites of mefruside in man.

The metabolism of mefruside in the rat was studied by Duhm and coworkers (1967). Two urinary metabolites, which were formed by oxidation of the 5-C atom of the tetrahydrofuran ring of mefruside, constituted together ca. 35% of the dose in this species. These metabolites appeared to be connected with each other through a lactone-hydroxy acid equilibrium (Fig. 5.1). The reaction was shifted completely to the lactone side under acidic conditions, e.g. pH 2, and to the open acid side at alkaline pH, e.g. 11, the rates of ring-closure and hydrolysis being pH-dependent (Pütter and Schlossmann, 1972; Schlossmann and Pütter, 1973). In aqueous buffers of pH 7-8, interconversion of both compounds was unmeasurably slow, but it became highly accelerated by addition of rat plasma or rat liver homogenates. Also in vivo, in dogs, rapid turnover of the two metabolites has been observed (Schlossmann and Pütter, 1973).

The possible occurrence of the above metabolites in man called our attention especially, because both compounds had been reported to evoke the same diuretic effects as the parent drug, after i.v. administration to rats (Meng and Kroneberg, 1967), so might be responsible too for diuretic activity. The aim of the investigations described in this chapter was, therefore, to develop an assay for these mefruside metabolites. Due to their particular chemical constitution, care had to be taken to avoid unwanted interconversion of the compounds in vitro prior to their separation.
MATERIALS AND METHODS

Drugs

5-Oxo-mefruside, 4-chloro-N1-methyl-N1-(tetrahydro-2-methyl-5-oxo-2-furanyl) methyl-1,3-benzenedisulfonamide (Fig. 5.1), and the internal standard for gas chromatographic analysis (the same compound as used in Chapter 4) were supplied by Bayer (Wuppertal, G.F.R., courtesy of Dr. H. Horstmann). The synthetic 5-oxo-mefruside sample contained 1.25 wt.% of mefruside, as evidenced by thin layer chromatography (system described in Materials section of Chapter 4, RF mefruside = 0.49, RF 5-oxo-mefruside = 0.43) and by gas chromatography, according to the method of Chapter 4. No attempt was made to separate the lactone from mefruside on a preparative scale, because both compounds were well-separated on the gas chromatographic column (see next section). Therefore mefruside did not influence peak area measurement of 5-oxo-mefruside and, in analysing unknown concentrations, only a small correction for the weighed amount of the latter needed to be made. The conformation of the supplied 5-oxo-
mefruside was checked by nuclear magnetic resonance measurements and elemental analysis (C, H, N)*, which yielded data confirming the lactone structure, and in agreement with literature (Horstmann et al., 1967). Stock solutions of 5-oxo-mefruside were prepared in 0.1 M aqueous HCl and diethylether, in concentrations of 50 μg/ml and dilutions thereof.

The hydroxy acid analogue of 5-oxo-mefruside, 4-chloro-N1-methyl-N1-(2-methyl-2-hydroxy-4-carboxybutyl)-1,3-benzenedisulfonamide, was obtained by dissolving 5-oxo-mefruside (50 μg/ml) in 0.1 M NaOH, in which very rapid hydrolysis, with a first-order half-life of 20-25 seconds, has been found (Schlossmann and Putter, 1973). Completeness of reaction was checked after 15 minutes by extracting aliquots (0.1 ml; 5 μg) added to 2 ml of Sörensen phosphate buffer pH 7.4, twice with 10 ml of diethylether. At this pH, only the lactone form can be extracted (as will be shown in the Results section). No trace (>10 ng) was found with the gas chromatographic method described in this chapter, indicating complete formation of the hydroxy acid.

**Derivatization and gas chromatography**

With some modification, the gas chromatographic method reported for the assay of mefruside in body fluids (Chapter 4, Fleuren et al., 1979) could be used. In short, this method consists of extraction of drug and internal standard at pH 7.4 into diethylether, re-extraction into 0.1 M aqueous sodium hydroxide, and ion-pair extraction with tetrahexylammonium-counterion into dichloromethane, under simultaneous methylation with iodomethane, during 15 minutes at room temperature ("extractive methylation", principle outlined in Chapter 2). After evaporation of the organic phase, the methylated derivatives are re-dissolved in n-hexane, in order to remove the co-extracted tetrahexyl-ammonium iodide, which is very sparingly soluble in this solvent, so that strong tailing of this substance on the gas chromatographic column is avoided. The methyl derivatives are thereafter concentrated in ethylalcohol, prior to injection into the gas chromatograph.

5-Oxo-mefruside and its hydroxy acid analogue were extracted as described in next sections of this chapter. Furthermore, the assay was modified with respect to the original procedure for mefruside in the dissolution step with n-hexane. Intensive sonification, twice for 15 min., with 5 ml of n-hexane appeared to be necessary for reproducible and complete dissolution of the more polar metabolites. The wash of the hexane layer with aqueous silver sulphate solution, by which the background

* Performed at the Department of Organic Chemistry, University of Nijmegen, Nijmegen, The Netherlands
signal of the gas chromatogram became reduced (Chapter 4), could not be employed for 5-oxo-mefruside, as this step removed its methylated derivative almost completely.

All reagents used, gas chromatographic equipment, and other apparatus were the same as described in Chapter 4. The methylated derivatives of the internal standard, mefruside and 5-oxo-mefruside eluted from the gas chromatographic column (3% SE30, 265°C) at retention times of 2.8, 4.1 and 6.3 minutes, respectively. The derivative, formed by methylation of the hydroxy acid analogue of 5-oxo-mefruside had an identical retention time as the derivative of the latter. This was not unexpected because both compounds pass through an alkaline layer (0.1 M NaOH) prior to methylation, so that the lactone is transformed completely to the free acid form, and a common species is generated. A discussion of the structure of this derivative during gas chromatography will be given in the Results section of this chapter.

**pH-dependent distribution between aqueous and organic phases**

*Lactone partitioning*

Into glass-tubes, containing 2 ml of aqueous buffers varying from pH 2 to pH 10 (citrate-phosphate-borate, 0.1 M, according to Teorell and Stenhagen), were pipetted 10 ml portions of a 0.5 µg/ml solution of 5-oxo-mefruside in freshly distilled diethylether. The tubes were well-closed and vigorously shaken during 5 minutes. After brief centrifugation, as much as possible of the diethylether layer was transferred into another tube. The samples containing the buffers of highest pH were handled first in order to minimize lactone-hydrolysis. At pH 11, the half-life of lactone-hydrolysis is 27 min (Schlossmann and Pütter, 1973), which implies that after 5 min. in such solution 88% of the drug is still in the lactone form, i.e. a loss of 12%. Because the highest pH we employed was a full pH unit lower (pH 10), we estimated a loss of less than 5% at that pH, which should have an insignificant influence on the shape of the distribution curve.

*Hydroxy acid partitioning*

0.1 Ml portions of a 50 µg/ml solution of the hydroxy acid analogue of 5-oxo-mefruside in 0.1 M NaOH were added to 1.9 ml portions of buffer of pH 2-10 (same buffer as described above). After mixing, 10 ml portions of freshly distilled diethylether were added and the tubes were vigorously shaken for 5 minutes. After short centrifugation (1 min), as much as possible of the organic layers was transferred into new tubes. For samples containing the buffers of lowest pH this separation was performed at once in order to minimize lactone formation. The half-life of ring-closure at pH 2 is 3.3 hours (Schlossmann and Pütter, 1973), which means that 98.3% of drug is still in the free acid form after 5 minutes at this pH, so that the
observed distribution at the lowest pH values too should be a true reflection of the lipophilicity of the pure open acid species.

Measurement of extraction recovery
To calibrate the above partitioning studies, known amounts of lactone (0.1-0.5 µg) and free acid (0.05-5 µg) were dissolved in diethylether (10 ml) and 0.1 M NaOH (2 ml), respectively. The sodium hydroxide layers were used directly, the diethylether layers extracted first with 2 ml of 0.1 M NaOH for 5 minutes, and then used in the extractive methylation, together with internal standard (5 µg in 0.1 ml of 0.1 M NaOH). The percentage of extraction at each pH was calculated by comparison of the ratios of the peak areas of the drugs and internal standard.

Procedure for the extraction of 5-oxo-mefruside and hydroxy acid analogue from biological samples
In order to determine 5-oxo-mefruside, 2 ml of plasma, 0.5-1 ml of packed red cells (1-2 ml of whole blood), or 1 ml of urine were extracted at pH 7.4 twice with 10 ml of diethylether under the same conditions including addition of internal standard, as described for mefruside in Chapter 4.

After this extraction of 5-oxo-mefruside, the same biological samples served to determine the hydroxy acid species. Plasma (2 ml) or urine (0.25-1 ml) was adjusted at pH 2 with a few drops of 2 M aqueous hydrochloric acid, and extracted, after renewed addition of internal standard, twice with 10 ml of diethylether for 5 min. Red blood cell or whole blood samples, however, extracted at pH 2, yielded a very high background signal in their gas chromatograms and in addition unknown disturbing peaks. Therefore, the hydroxy acid in these samples was converted into the lactone form by leaving the tubes at pH 2 for 24 hours or longer. Subsequently, the samples were adjusted again at pH 7.4 with solid NaHCO₃ (100-200 mg/sample), internal standard and buffer of pH 7.4 were added until a final volume of 2.5 ml in the tube and the lactone was extracted in the same way as described at the beginning of this section. Previously, we had verified that ring-closure was completed under these conditions, by comparison with known amounts of 5-oxo-mefruside. (Moreover, in assaying urine concentrations of the hydroxy acid, the same quantitative results were found from the lactone-conversion procedure as from direct extraction at pH 2.) Parallel to each series of biological samples, two or three standards of 5-oxo-mefruside and its hydroxy acid counterpart, added to a corresponding volume of pH 7.4, were extracted at pH 7.4 or pH 2, derivatized and submitted to gas chromatography, in order to check the overall procedure. For this calibration, known amounts bracketing the concentration range of the unknown samples were taken, e.g. 0.5, 1 and 2.5 µg of 5-oxo-mefruside in the urine assay.
Sampling of blood and urine, in vitro distribution between plasma and red cells

The method of collection of venous blood from human subjects was the same as described for mefruside in Chapter 4. Shortly summarized, heparinized 7 ml blood samples were centrifuged at 3000 r.p.m. for 3 minutes at room temperature, immediately after vein puncture, and the plasma was rapidly taken apart. Because the equilibration of mefruside between plasma and red cells was unmeasurably rapid, and the position of the final equilibrium temperature-dependent, the decrease in blood temperature inherent to routine treatment of blood samples (vein puncture, centrifugation) caused an altered red blood cell-plasma concentration ratio of this drug (Chapter 4; Fleuren et al., 1979). A similar phenomenon could be possible for 5-oxo-mefruside and its hydroxy acid counterpart. Therefore, partitioning of these two compounds between plasma and erythrocytes was studied in fresh human blood, according to the methods described in Chapter 4. Thus, the rate of distribution at 37°C, and the difference between plasma concentrations present at in vivo temperature (37°C) and those found after routine treatment of the blood (27.5°C) were determined. A complicating factor arose from interconversion of the lactone and the open acid by incubations in fresh human plasma. Although different in extent for each plasma sample, hydrolysis of the lactone could account for as much as 25% of the added amount, but ring closure of the hydroxy acid was always less than 5% (at plasma concentrations of 40 μg/ml). This pattern was qualitatively in agreement with observations of Schlossmann and Pütter (1973) for the interconversion of the two species in rat plasma. However, because interconversion in human plasma appeared to be finished after 15-30 minutes, we pre-incubated the two substances during 30 minutes in plasma at 37°C prior to the red blood cell partitioning experiments, and measured the concentrations of the drug under study both in plasma and red cells. The concentrations employed in the present in vitro studies are indicated in the Results section of this chapter.

Urine from human experiments was adjusted to pH 8 with a few drops of 5M NaOH, prior to extraction. Care was taken to mix the contents of the tube vigorously during pH adjustment, in order to prevent hydrolysis of 5-oxo-mefruside.

Comparison of presumed metabolite with synthetic 5-oxo-mefruside by gas chromatography - mass spectrometry

Electron impact mass spectra of the methylated derivatives of synthetic 5-oxo-mefruside and mefruside were compared with that of the suspected metabolite by means of a LKB 9000 gas chromatograph-mass spectrometer combination (LKB, Bromma, Sweden), at an accelerating potential of 20eV, trap current of 60 μA and ion source temperature of 260°C. For gas
chromatographic separation a glass column (1.5 m x 3 mm I.D.) was used, packed with 3% OV101 on Gas Chrom Q, 100-120 mesh (Applied Science Labs., State College, Pa., USA), at an oven temperature of 255°C. The retention times of the methylated derivatives of internal standard, mefruside, and 5-oxo-mefruside were 8.5 min, 13 min and 18 min, respectively.

RESULTS AND DISCUSSION

Drug partitioning

The distribution of 5-oxo-mefruside and its open-chain analogue between organic phase and aqueous buffer of varying pH is shown in Fig. 5.2. The plots indicate that the lactone can be recovered in the ether layer by a single extraction already with 87% yield from pH 2-8.5, similarly to extraction of mefruside, whereas the carboxylic acid can only be obtained in high yield (76%) below pH 3, and not at all extracted above pH 7. This latter

![Figure 5.2](image)

Plots showing the percentage of extraction of mefruside, 5-oxo-mefruside and the hydroxy acid metabolite against the pH of the aqueous phase.
detail was checked by separate extractions at pH 7.4 (not shown here), employing as aqueous phases both buffer alone, buffered plasma, red cells and urine. These experiments confirmed that the quantity of the open acid compound, recovered in the organic phase by two subsequent extractions with diethylether at this pH, was not detectable in our gas chromatographic assay, viz. < 10 ng on a total amount of 5 µg. This means that the percentage of hydroxy acid extracted at pH 7.4 is even lower than 0.2%. In this way, the two structural analogues were separated prior to following steps of analysis.

Gas chromatographic determination of 5-oxo-mefruside and its open acid counterpart in plasma, red blood cells and urine

Calibration graphs, prepared by adding known amounts of the lactone and the open acid to blank human plasma, urine and red cells, and plotting, after gas chromatographic analysis, peak area ratios of drugs and internal standard against concentration, were linear and passed through the origin. The standard deviation of the whole procedure, determined from repeated assay of the same samples was ca. 5% (n = 10) for both compounds at concentrations between 0.05-10 µg/sample. The lowest concentration which could accurately be analysed (i.e., with the above standard deviation) was approximately 25 ng/sample, whereas amounts down to 5-10 ng in biological extracts could still be detected. The recovery of 5-oxo-mefruside by two extractions with diethylether at pH 7.4 was 98.5 ± 1% (mean ± S.D., n = 4) in the concentration range investigated, independent of the choice of buffer alone, plasma, red cells or urine as the aqueous phase. The corresponding figure for extraction of the hydroxy acid analogue at pH 2 was 94 ± 2% (mean ± S.D., n = 4).

Identification of mefruside metabolites in biological samples

The gas chromatograms, obtained by analysis of urine from human subjects who had ingested an oral dose of mefruside (25 or 50 mg, as described in Chapter 14) showed a large peak eluting after that of mefruside itself, with a retention time of 6.3 minutes (Fig. 5.3), which was the same as that of the methylated derivative of synthetically prepared 5-oxo-mefruside. The peak was present already after extraction of urine at pH 7.4, but became larger when urine had been extracted at pH 2. This extra amount could also be recovered by extraction at pH 7.4, if the urine previously had been allowed to stand at pH 2 for several hours.

The electron impact mass spectrum of the presumed metabolite, extracted from human urine and separated by gas chromatography, was identical with that of the pure reference compound. The mass spectra of the methyl derivatives of mefruside and 5-oxo-mefruside are shown in Fig. 5.4. The fragmentation of both compounds is consistent with the scheme of
**Figure 5.3**

Typical gas chromatogram showing the presence of mefruside (0.15 μg/ml) and 5-oxo-mefruside (2.7 μg/ml) in human urine, analysed 5 hours after intake of a 50 mg dose of mefruside by a normal human subject (i.st. = internal standard).

**Fig. 5.5.** After being split off, the tetrahydrofuran ring of both mefruside and its oxidized metabolite appears to be relatively stable, as the m/e 85 and 99 fragments are very abundant. It can be seen, that a number of peaks in the upper panel is shifted to the right with respect to the mefruside spectrum with 14 m/e units, viz. 99 vs 85, 142 vs 128, and 381 vs 367, which dif-

**Figure 5.5**

Common mass fragmentations of the dimethylated derivatives of 5-oxo-mefruside (I, M.W. = 424, $^{35}$Cl), mefruside (II, M.W. = 410, $^{35}$Cl), and a straight-chain analogue (III, M.W. = 384, $^{35}$Cl).

A. Presumably formed after H-shift from m/e 44 fragment  
B. Base peak
Figure 5.4
Electron-impact mass spectra (20 eV) of the methylated derivatives of 5-oxo-mefruside and mefruside, after gas chromatographic separation.
ferences are clearly attributable to replacement of two hydrogen atoms by oxygen at the C-5 atom of the tetrahydrofuran moiety. Furthermore, the fragmentation pattern of the two compounds is closely comparable with that of a structural analogue with a straight chain (III in Fig. 5.5), which is used as the internal standard in the gas chromatographic assay of mefruside and the metabolites described in this chapter.

Only the lactone form of both occurring oxidized metabolites of mefruside has been drawn in Fig. 5.4. The thermodynamic equilibrium between these two species lays completely at the opened-acid side at alkaline pH (Schlossmann and Pütter, 1973; this chapter, Materials and methods section). There can be no doubt, therefore, that any derivative, formed by extractive methylation of either the lactone or the open acid as starting substance, existed in the open chain form exclusively, prior to injection into the gas chromatograph. Whether two, three or even four methyl groups have been introduced at that stage is not known. There is, however, besides the excellent agreement of the mass fragmentation pattern and molecular formula depicted in Fig. 5.4, another argument to favour the view that during gas chromatography the methylated derivative possesses the structure shown. It is well known that, by heating, γ-hydroxy carboxylic acids and their ester (and even ether) derivatives are readily converted to the corresponding lactones (Köper, 1963). Operated at a temperature of 300 °C, the injection port of the gas chromatograph must amply have provided such condition. It must be concluded, therefore, that both metabolites of mefruside, viz. the lactone and the open acid, are chromatographed as the same methylated derivative, with the formula depicted in Fig. 5.4.

Duhm and coworkers (1967) have proposed N-demethylation of mefruside or its metabolites in the rat to account for a small fraction of dose, leading to carbon dioxide in expired air after administration of the methyl-Cl4-labelled drug. The methylation which is employed in the present assay could have masked possible biological formation of N1-demethylated 5-oxo-mefruside or its hydroxy acid congener. The methylated product of this metabolite would be indistinguishable from that originating from 5-oxo-mefruside (or open acid analogue) itself. We were able to exclude already the presence of detectable amounts of demethylated mefruside in human body fluids by the use of propyliodide as the derivatizing agent (Chapter 4; Fleuren et al., 1979). In an analogous way, we now carried out propylation of both urine, plasma and red cell extracts from several human subjects, and did not find a trace of a substance with a gas chromatographic retention time other than that belonging to the propylated derivative of 5-oxo-mefruside itself. In this way, the specificity of the assay for 5-oxo-mefruside and its open acid counterpart was considered to be affirmed.
In vitro distribution of mefruside metabolites between plasma and erythrocytes

5-Oxo-mefruside and its hydroxy acid analogue equilibrated instantaneously between plasma and red cells of human blood. Thus, no difference was observed in the red cell-plasma concentration ratios at 37 °C, whether the blood had been centrifuged immediately after mixing red cells with plasma, or at 1.5, 5, 15 and 45 min after the start of the incubation.

The lactone and the open acid differed greatly in their extent of red cell partitioning. While the lactone reached concentrations in erythrocytes which were ca. 20 times higher than those in plasma, the hydroxy carboxylic acid concentrations in red cells were only one-tenth of those in plasma, at a whole blood concentration of 8 µg/ml for both experiments. For this reason, only the red cell uptake of the lactone was subjected to further investigation. The red cell-plasma concentration ratio appeared to be constant in the concentration range employed, 2-15 µg/ml whole blood, and mean ratios of 18 and 21 were found from incubations at 37°C in blood from two human subjects.

When blood incubated with the lactone at 37°C was centrifuged at room
temperature for 3 min (resulting in a blood temperature of 27.5°C, as described in Chapter 4), the plasma concentrations of the drug were 96.5 ± 1.5% (mean ± S.D., n = 6) of the values obtained, when the whole procedure was carried out at 37°C. It was concluded therefore that plasma concentrations of 5-oxo-mefruside, found after immediate centrifugation of blood following vein puncture, differ by only ca. 3.5% from the concentrations actually present at in vivo temperature. Because both 5-oxo-mefruside and mefruside (Chapter 4) are rapidly taken up by red blood cells, we were interested to see if the binding of the metabolite would influence that of the parent drug. Fig. 5.6 shows that the erythrocyte-plasma concentration ratio of mefruside, incubated in whole human blood, decreases with increasing concentration of 5-oxo-mefruside. Although the interaction seems of little importance at whole blood concentrations of the lactone below 1 μg/ml, the effect visible at higher concentration might also play in vivo. Some evidence for displacement of mefruside from its red cell binding sites in vivo can be found in Chapter 14, which deals with the pharmacokinetics of mefruside in man.

SUMMARY

A gas chromatographic method was reported for the quantitative determination of two metabolites of mefruside, viz. 5-oxo-mefruside (mefruside-lactone) and its hydroxy carboxylic acid analogue, in human body fluids. Use was made of extractive methylation as derivatization technique, and quantitation was achieved, with help of a suitable internal standard, by means of a nitrogen-sensitive detector.

Because the two metabolites were linked chemically through a lactone-open acid equilibrium, interconversion prior to their separation had to be avoided. A pH-partitioning study was performed to find optimal separation conditions. The lactone could be extracted quantitatively at pH 7.4, without any trace of co-extracted hydroxy acid. The latter was extracted either at pH 2 directly (in case of plasma and urine), or after conversion to the lactone at pH 7.4 (in case of red cells or whole blood). Concentrations down to 25 ng/sample of both compounds could be analysed with a standard deviation of 5%.

5-Oxo-mefruside and its open chain counterpart were identified as human metabolites by gas chromatography-mass spectrometry. The mass spectrum of authentic 5-oxo-mefruside was identical to that of the compound present in human urine.

The two metabolites of mefruside equilibrated instantaneously between red cells and plasma in vitro. At 37°C, the red cell-plasma concentration
ratio was 20 for the lactone, but only 0.1 for the open acid compound. It was shown by in vitro incubations, that concentrations of 5-oxo-mefruside in plasma, obtained by a 3 min centrifugation of blood samples at room temperature, immediately after vein puncture, are ca. 3.5% lower than the values actually present at in vivo temperature. 5-Oxo-mefruside was able to displace mefruside from its red blood cell binding sites in vitro.

REFERENCES


CHAPTER 6

DIFFERENCE POTENTIOMETRIC METHOD FOR DETERMINING DISSOCIATION CONSTANTS OF VERY SLIGHTLY WATER-SOLUBLE DRUGS, APPLIED TO THE SULFONAMIDE DIURETIC CHLORTHALIDONE

INTRODUCTION

The dissociation constant of a drug is an important parameter in pharmacokinetic and pharmacodynamic investigations. Once the pKa is known, the degree of dissociation of the compound at physiological pH is easily derived and, as it may be assumed that in general only unionized molecules pass readily across biological membranes (see e.g. Goldstein, 1974), predictions concerning the access to tissue sites of interest can be made. Besides, the pKa has value in the estimation of the lipophilicity of a compound from its partitioning between an organic solvent and water: true partition coefficients can be calculated from the apparent values at any arbitrary pH, as outlined in Chapter 2.

Chlorthalidone contains two weakly acidic groups: one sulfonamide and one oxo-isoindolin (acid amide) moiety (Fig. 6.1). Although the latter was not expected to contribute to dissociation of the drug at acid or neutral pH, the former could influence back-resorption of the drug in the renal tubules, when urine of pH 8 is produced. In that situation a pH-dependent urinary excretion should become evident. This aspect gains quantitative importance, when it is realized that normally ca. 70% of an available dose of chlorthalidone is excreted unchanged in the urine (Fleuren et al., 1979; Chapter 9). The present study was undertaken because no pKa value for chlorthalidone was available from the literature.

![Figure 6.1](image)

Structural formula of chlorthalidone.
Various methods to determine dissociation constants of acids and bases are in common use. These were comprehensively evaluated by Albert and Serjeant (1962). These authors stated that both by potentiometry and by ultraviolet spectrophotometry, which belong to the more convenient methods, accurate results can be obtained, although the latter is more laborious.

One of the premises for a conventional potentiometric titration is that the concentration of the weak acid or base must be so high that the amount of titrant, needed to titrate the solvent, is negligible in comparison with the amount necessary to protonate or deprotonate the dissolved compound. This condition could not be fulfilled at present, because the maximum solubility of chlorthalidone in neutral aqueous solutions was reported as ca. 0.12 mg/ml (Stenger et al., 1959), equivalent to $3.45 \times 10^{-4}$ M. In such a case a difference titration can be conducted, provided that the amounts of strong acid and base, with which the solution of the compound with unknown pKa and its blank (solvent alone) are titrated, are measured accurately. Moreover, the pH measurement must be of high precision and very reproducible. In order to meet these requirements use was made of an automatic potentiometric titration equipment, containing a precisely operating micro-burette, motor-driven, and a high-resolution digital voltmeter, connected to a low drift pH meter. The apparatus has been used previously to determine pKa values and numbers of titratable groups of purified proteins (de Bruin et al., 1969; Janssen et al., 1970).

EXPERIMENTAL

An aqueous solution of 0.1 M KCl served as blank. A saturated solution of chlorthalidone in this solvent was prepared by vigorously stirring ca. 8 mg of the crystalline drug in 50 ml at room temperature overnight. After filtration, the concentration, determined in triplicate by gas chromatography (Chapter 3; Fleuren and van Rossum, 1978), was $125.2 \pm 0.7 \mu g/ml$ (mean $\pm$ S.D.), equivalent to $3.695 \times 10^{-4}$ M. Aqueous hydrochloric acid and sodium hydroxide solutions, 0.0604 M, were diluted by weighing with four volumes of distilled water and bubbled with nitrogen gas to remove carbon dioxide. The titrant was added stepwise by the use of a micro-burette (Metrohm, Zürich, Switzerland), dosing each minute 0.01 ml with an accuracy of $\pm$ 0.1%. The titrations were conducted on the chlorthalidone solution (4.00 ml) and on the solvent alone (4.00 ml), at 25°C and under nitrogen, in both directions of the pH range 3.5-10.6. Two runs with HCl (exp. B, D) and two with NaOH (exp. A, C) were carried out. The actual pH ranges were: 3.47-10.28 in A, 8.55-10.58 in B,
8.10-10.50 in C and 6.61-10.45 in D. A pH meter in combination with a
glass and a calomel electrode was used (all from Radiometer, Copenhagen,
Denmark: types PHM 26, G 202 B, and K 401 resp.). A high-resolution
digital voltmeter (type LM 1867, Solartron, Peekel, Rotterdam, The
Netherlands) was connected with the pH meter. The pH was measured 10
times after each addition of HCl or NaOH and the mean of these readings
was taken. In this way very reproducible values, including the third decimal
figure, are obtained (van Os et al., 1972). Detailed descriptions of this
automatic potentiometric equipment have been published by Janssen et al.,
(1970) and van Os et al., (1972). To calibrate the pH meter, phthalate buffer
(pH 4.008) and phosphate buffer (pH 6.865) were used.

RESULTS AND DISCUSSION

Typical titration curves are shown in Fig. 6.2. Equal volumes of 0.1 M
KCl, with and without chlorthalidone, were titrated with dilute sodium
hydroxide from pH 3.5 towards pH 10.3. A larger number of additions of
alkali is needed to increase the pH of the drug solution in the region, where
the drug becomes deprotonated. The difference at each pH value between
the number of additions of the two titration runs clearly represents the
quantity necessary for titration of the drug itself. When these differences
are plotted versus the pH a difference titration curve is obtained. For a
weak acid the titration curve obeys the familiar Henderson-Hasselbalch
equation:

\[ pH = pK' + \log \frac{\alpha}{1-\alpha} \]  
(Eq.6.1)

where \( \alpha \) represents the degree of dissociation of the acid group and \( pK' \) its
apparent dissociation constant, which becomes equal to the thermodynamic
dissociation constant, \( pK_a \), at infinite dilution. At higher ionic
strength, the following relationship is valid:

\[ pK' = pK_a - \log \frac{\gamma_{A^-}}{\gamma_{HA}} \]  
(Eq.6.2)

in which \( \gamma_{A^-} \) and \( \gamma_{HA} \) are the activity coefficients of, respectively, the
ionized and unionized forms of the acid. The activity coefficients can be
calculated according to the Debye-Hückel equation (see e.g., Adrien and
Serjeant, 1962): for log $\gamma_{A^-}/\gamma_{HA}$ a value of -0.11 was obtained. By using this correction, the pKa is readily obtained if the experimental value pK’a is known.

When a strong base is added, an equivalent amount of protons is lost by the weak acid. Then $\alpha$ can be written as $\alpha = Z/A$, where $Z$ represents the
amount of strong base added at any moment of the titration and A the amount added at the titration endpoint. When polyacids are titrated, we will have the relation:

\[ Z = \sum_{i=1}^{n} A_i \cdot \alpha_i \quad \text{(Eq.6.3)} \]

where \( i \) denotes the identity of a specific weak acid group and \( n \) is the total number of classes of weak acid groups.

Chlorthalidone contains two acidic moieties, one sulfonamide and one oxo-isoindolin group, potentially dissociating in the pH range under study. Therefore, \( A_1 = A_2 \) and

\[ Z = A_1 (\alpha_1 + \alpha_2) \quad \text{(Eq.6.4)} \]

By applying Equation 6.1, we can write:

\[ Z = A_1 \left[ \frac{\text{pH}-\text{pK}_a}{10} \frac{\text{pH}-\text{pK}_a}{1 + 10} \right] + \left[ \frac{\text{pH}-\text{pK}_a}{10} \frac{\text{pH}-\text{pK}_a}{1 + 10} \right] \quad \text{(Eq.6.5)} \]

The titration data were analysed by means of a non-linear least squares regression analysis program.* In every curve-fitting procedure the correct assignment of independent and dependent variables is of essential importance to obtain unbiased estimates of the unknown parameters (Perrin et al., 1974). The pH was chosen as the independent variable, because its measurement was highly reproducible (see experimental section), such that there existed, apart from a potential calibration error, which could only influence the absolute pH scale, a very small error within each experiment. For the dependent variable \( Z \) a constant error of 0.1, expressed as additions of strong base, was taken as this was the read-off uncertainty of the differences between the titration curves of the chlorthalidone solution and the blank, see e.g. Fig. 6.2.

* Farmfit, a digital computer program in use at the Computer Centre of the University of Nijmegen. Details in Chapter 1.
At first, the data points were fitted according to the first term of Equation 6.5 only, assuming that the dissociation of the second acid group was negligible (if pH \( < pK'a_2 \), the second term approximates zero). It came out, however, that much better fits were obtained when also the second term was taken into account. For this purpose the value for pK'a_2 was not left free in the least squares regression analysis, because a too small number of data points was available in the high pH region, only up to pH 10.6, which would introduce large errors in the estimates of the other parameters, A_1 and pK'a_1. Preset values of pK'a_2 were systemically changed, until the minimum of the sum of weighed squared deviations had been found. The results of these calculations are included in Table 6.1 (pK'a_2 has been denoted k_2). It has to be noticed that also for A_1 the best estimate was searched by the fitting procedure, because this parameter, when it would be fixed at its theoretically calculated value (12.24 additions of strong base), would influence the pK'a_1 estimate with possible errors in the concentration and pipetted volume of the chlorthalidone solution and in the normality and volume of the titrant.

![Potentiometric Titrations](image)

**Figure 6.3**

Computer-fitted difference titration curve of chlorthalidone. From four experiments the following estimates resulted for pK'a_1: 9.228 (A), 9.287 (B), 9.210 (C), 9.235 (D), yielding a mean apparent dissociation constant of 9.24±0.02 (± S.E.M.) at 0.1 ionic strength and 25°C.
TABLE 6.1

Superiority of the computer fits of difference titration curves of chlorthalidone by taking into account the dissociation of two weak acid groups (left part) instead of one (right part)

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK'(a_1)</td>
<td>9.228</td>
<td>9.287</td>
<td>9.210</td>
<td>9.235</td>
<td>9.24 ± 0.02</td>
</tr>
<tr>
<td>(A_1)</td>
<td>12.15</td>
<td>12.33</td>
<td>13.27</td>
<td>12.67</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>(k_2)</td>
<td>9.94</td>
<td>11.11</td>
<td>11.55</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>12.6</td>
<td>14.3</td>
<td>8.1</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>pK'(a_1)</td>
<td>9.35</td>
<td>9.56</td>
<td>9.27</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td>(A_1)</td>
<td>14.43</td>
<td>14.16</td>
<td>14.17</td>
<td>12.96</td>
<td></td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>96.6</td>
<td>210</td>
<td>67.5</td>
<td>111</td>
<td></td>
</tr>
</tbody>
</table>

\(pK'\(a_1\)\) = apparent first dissociation constant, \(A_1\) = total amount of base at first titration endpoint, \(k_2\) = optimally fixed value for the dissociation of the second acid group, \(\chi^2\) = sum of weighed squared deviations of the experimental data from the fitted curve.

\(\chi^2\) has the tabulated level of significance at \(P = 0.05\), was 39.44, 40 and 37 in the experiments A, B, C, and D, respectively.

The thermodynamic dissociation constant, \(pK_a\), is larger than the \(pK'\(a_1\)\) by a factor 0.11, due to the contribution of activity coefficients. Therefore, \(pK_a = 9.35 ± 0.02\) (mean ± S.E.M.).

Fig. 6.3 shows a typical difference titration curve, fitted according to Equation 6.5 (at fixed optimal \(pK'\(a_2\)\) values). The definite decision in favour of such fit over that based upon ionization of only one acid group was made by several criteria: 1) visual inspection of the plots, 2) symmetry of the residuals around the fitted curves, 3) calculation of the sum of weighed squared residuals (as \(\chi^2\)). Table 6.1 compares the \(\chi^2\)-values for both procedures and indicates the superiority of curve-fitting according to two titratable groups in all four experiments. In addition, it can be seen that the scatter of the \(pK'\(a_1\)\) estimates in the left part is much smaller than that in the right part. Also the estimates of \(A_1\) at the left correspond better with the value calculated on the basis of the total amount of chlorthalidone in the titration vessel (equivalent to 12.24 additions of strong base), indicating that actually one proton-donor group was dissociated completely at this stage.

The variance of the four \(pK'\(a_1\)\) estimates exceeds the within-experiment error (the computer estimated relative errors ranged from 0.0-0.1%). This points to minor systematic differences between the four experiments, e.g. caused by the pH meter calibration. However, the differences are small
enough to trust upon the $pK'a_1$ (25°C) = 9.24 ± 0.02 (mean ± S.E.M.) as a reliable operational ionization constant, suitable for use as a physico-chemical parameter in pharmacological studies. When the correction for ionic strength is carried out with Equation 6.2 the thermodynamic dissociation constant is calculated at $pK'a_1 = 9.35$ (25°C).

Determination of dissociation constants of very slightly water-soluble drugs appears to be performed conveniently and reproducibly by the method described, and no corrections for titrant consumption by the solvent need to be made. The technique was applied also to the analgesic ibuprofen ($pK_a = 4.4$, preliminary estimate) in our laboratory (van Ginneken, 1976), indicating that potentiometric difference titrations can be conducted in a wide area of pharmaceutical substances.

**SUMMARY**

A renewed application of potentiometric acid-base titrations is described, by which dissociation constants of practically water-insoluble drugs can be measured accurately. The method makes use of the difference in the amount of titrant between a suitable aqueous solvent and a solution of the drug in that solvent. Such potentiometric difference titrations were conducted on a $3.7 \times 10^{-4}$ M solution of chlorthalidone in 0.1 M aqueous KCl, in the pH 3.5-10.6 range, at 25°C. Non-linear least squares regression analysis was applied to the data. From four determinations, a value of $9.24 ± 0.02$ (mean ± S.E.M.) resulted for the apparent dissociation constant of the first acid group of chlorthalidone. The thermodynamic dissociation constant was calculated at $pK'a_1 = 9.35$ (25°C) by using a correction for activity.

**REFERENCES**


SECTION III

PHARMACOKINETICS OF CHLORTHALIDONE
INTRODUCTION

The phthalimidine derivative chlorthalidone (Hygroton®) was introduced in the years around 1960 as a diuretic with a long duration of action, lasting for up to 48-72 hours (Reutter and Schaub, 1959; Ford, 1960; Mach and Veyrat, 1960). The overall type of diuresis is comparable with that observed from the thiazide diuretics, i.e. chlorothiazide, hydrochlorothiazide and congeners (Peters and Roch-Ramel, 1969). As outlined in Chapter 1, the similar shape of the dose-response curves of all members of the benzothiadiazine family has led to acceptance of a common mechanism of action for them. Accordingly also chlorthalidone has been classified within this group.

Despite extensive use of chlorthalidone in pharmacotherapy, very little was known, up to 1974, about its fate in the human body. Pulver et al. (1959) administered the drug to rabbits and dogs, and observed accumulation and retention in renal tissue. Measurable concentrations in blood were found only after eight hours following oral administration. These authors suggested, therefore, that slow and protracted absorption would be one of the reasons for the long lasting effect of chlorthalidone. However, Beisenherz et al. (1966), using C14-labelled chlorthalidone in rats, observed a much more rapid appearance of the drug in blood. In this species, the drug was extensively metabolized and excreted into the bile; the nature of the metabolites remained unclear. These workers were the first, who noticed uptake of chlorthalidone in red blood cells, in which the concentrations were 5-6 times higher than those in plasma. Their finding was confirmed by Tweeddale and Ogilvie (1974), who had modified the spectrophotometric assay of Pulver et al. (1959), but were unsuccessful with this method to measure plasma concentrations in the therapeutic concentration range.

The above-mentioned reports were available when our studies were initiated. During a preliminary study (Fleuren and van Rossum, 1975) a much more pronounced accumulation of chlorthalidone in human red blood cells was observed than that described earlier. The concentration in erythrocytes in vivo was at least 50 times higher than that in plasma, so that - in case of chlorthalidone pharmacokinetics - the erythrocytes must be
regarded as an important tissue compartment. An advantage of this tissue is that it is easily accessible, thereby providing a general model system for analysing the kinetics of drugs exhibiting tissue binding.

During the course of our investigations other workers have contributed also to knowledge of the human pharmacokinetics of chlorthalidone. The results of Beermann et al. (1975), Collste et al. (1976) and Riess et al. (1977) were generally in agreement with our findings and will be referred to at the appropriate places throughout this manuscript. Some discrepancy occurred between our results and those from the above-mentioned groups with respect to the observation of the differing elimination half-lives for plasma and red blood cell concentrations of chlorthalidone. As described in Chapter 9, this divergency can be explained by a different way of handling the blood samples, at which point certain precautions have to be taken. We made considerable efforts in this respect, resulting in a reliable method for determining chlorthalidone plasma concentrations, as is outlined in Chapter 3.

This chapter will present merely a general framework for the kinetics of chlorthalidone in man, especially focusing on the in vivo distribution of this drug between plasma and red blood cells. An attempt will be made to integrate the relationship between plasma and erythrocyte concentrations into one model, including all observed features simultaneously.

Details of the kinetics of absorption and elimination of the drug will be treated in depth in the next chapters of this thesis. Thus, information on the rate and extent of absorption, volumes of distribution and total plasma clearance of chlorthalidone can be found in Chapter 9. Urinary excretion and renal plasma clearance after different dose levels are dealt with in Chapter 10. The Chapters 11 and 12 will be devoted to the processes of biliary and salivary excretion, respectively. Chapter 13, finally, will describe accumulation of plasma and red blood cell concentrations of chlorthalidone during multiple dosing.

MATERIALS AND METHODS

Chlorthalidone was given in doses of 100 or 200 mg to 10 young, healthy male subjects varying in age from 22-25 years. The drug was administered in the morning after overnight fasting in the form of a tablet (Hygroton®). Blood samples were drawn by vena punction and heparinized, while simultaneously the urine was collected in separate portions over a period of at least 4 days. Plasma, urine and red cells were assayed with the gas chromatographic method described in Chapter 3. In vitro incubations of chlorthalidone in human blood were carried out by gently moving freshly
taken heparinized blood, spiked with the drug, in a waterbath of 37°C, under an open air atmosphere, during 1.5 hours. A pilot study proved this period to be sufficient to reach equilibrium distribution, as shown in Chapter 3.

RESULTS

Time course of chlorthalidone concentration in plasma and red blood cells

A rapid absorption of chlorthalidone into plasma was observed, with peak concentrations occurring at 1.5-3 hours after the dose. The decay thereafter was biphasic. Red blood cell concentrations of chlorthalidone reached their maximum much more slowly, i.e. 10-15 hours following administration, and declined thereafter mono-exponentially. The time of the maximum erythrocyte levels coincided with the end of the first phase of disappearance of plasma concentration of chlorthalidone. At that stage,

Figure 7.1
Pharmacokinetic profile of chlorthalidone in a human volunteer after an oral dose of 100 mg (Hygroton®). Concentrations were measured both in the blood plasma and in the erythrocytes. Plasma decay ($t_{1/2} = 46$ hr) was found to be considerably faster than erythrocyte decay ($t_{1/2} = 65$ hr).
Comparison of elimination half-lives in the plasma compartment with those in the erythrocytes in human volunteers after an oral dose of 100 or 200 mg chlorthalidone (Hygroton).  

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Body weight (kg)</th>
<th>Time period measured (hr)</th>
<th>Dose (mg)</th>
<th>( t_{1/2} ) plasma (hr)</th>
<th>( t_{1/2} ) erythrocytes (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J Bi</td>
<td>22</td>
<td>69</td>
<td>100</td>
<td>200</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>J Bi</td>
<td>22</td>
<td>69</td>
<td>80</td>
<td>100</td>
<td>42</td>
<td>52</td>
</tr>
<tr>
<td>D Bo</td>
<td>22</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>D Bo</td>
<td>22</td>
<td>80</td>
<td>200</td>
<td>200</td>
<td>42</td>
<td>64</td>
</tr>
<tr>
<td>M. Br.</td>
<td>23</td>
<td>81.5</td>
<td>100</td>
<td>100</td>
<td>45</td>
<td>66</td>
</tr>
<tr>
<td>M. Br.</td>
<td>23</td>
<td>81.5</td>
<td>200</td>
<td>100</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td>H F</td>
<td>25</td>
<td>67</td>
<td>80</td>
<td>200</td>
<td>34</td>
<td>58</td>
</tr>
<tr>
<td>V. Fl</td>
<td>23</td>
<td>68</td>
<td>100</td>
<td>100</td>
<td>30</td>
<td>51.5</td>
</tr>
<tr>
<td>O L</td>
<td>23</td>
<td>74.5</td>
<td>100</td>
<td>100</td>
<td>39</td>
<td>62</td>
</tr>
<tr>
<td>M R</td>
<td>23</td>
<td>68.5</td>
<td>100</td>
<td>100</td>
<td>44</td>
<td>68</td>
</tr>
<tr>
<td>T S</td>
<td>22</td>
<td>88</td>
<td>100</td>
<td>200</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>T S</td>
<td>22</td>
<td>88</td>
<td>100</td>
<td>100</td>
<td>55</td>
<td>69</td>
</tr>
<tr>
<td>T S</td>
<td>22</td>
<td>88</td>
<td>200</td>
<td>100</td>
<td>42</td>
<td>64</td>
</tr>
<tr>
<td>J W.</td>
<td>25</td>
<td>65</td>
<td>100</td>
<td>100</td>
<td>28</td>
<td>50</td>
</tr>
<tr>
<td>T B.</td>
<td>24</td>
<td>64</td>
<td>100</td>
<td>100</td>
<td>27</td>
<td>42</td>
</tr>
</tbody>
</table>

Mean value (SD)  

40 (8) 60 (8)

*The half-life values were calculated by regression analysis, based on at least five data points in the terminal part of the decay curves.*

the concentrations in the red blood cells were 50-100 times higher than those in plasma, and this ratio increased during the elimination phase. Consequently, longer elimination half-lives were obtained from red blood cell concentrations than from those in plasma. The final phase of a typical drug concentration curve in plasma and erythrocytes is shown in Fig. 7.1. Comparative data from ten subjects, studied after administration of single oral 100 and 200 mg doses, are given in Table 7.1. The mean half-life in this group for terminal plasma decay is 40 hours, and that for erythrocyte decay is 60 hours.

**Urinary excretion**

A detailed account of the urinary excretion of chlorthalidone in man is made in Chapter 10. In short, all urinary excretion rate plots were perfectly parallel to the time course of plasma concentrations, implying that the tubular secretion mechanism, by which all sulfonamide diuretics are excreted (Beyer and Baer, 1975; Chapter 1), remains far from saturated in the dose range employed. An example of an urinary excretion rate plot in a human subject is presented in Fig. 7.2. Cumulative renal excretion of un-
changed chlorthalidone amounted to ca. 25-50% of dose, as measured by extrapolation to infinite time after the actual period of assay.

**Distribution of chlorthalidone between plasma and red blood cells**

In the concentration range reached after oral administration of chlorthalidone the in vitro distribution is non-linear having an initial great rise in the erythrocytes/plasma ratio followed by a phase of more gradual accumulation in the red blood cells. See Figure 7.3a. A similar view is obtained from data of hypertensive patients (n = 20), who were on chlorthalidone
medication (Hygroton®, 100 mg/day) during 5 days or more. Despite the considerable scatter in this plot, which may be due to differences in the distribution ratio between individuals, the trend is essentially the same as that obtained from the in vitro experiments. See Figure 7.3b.

**Figure 7.3a**
Relationship between plasma concentrations and red blood concentrations of chlorthalidone *in vitro* after equilibration of the drug in whole blood of a healthy volunteer. The plot can be interpreted as consisting of a sum of two parts; the first part is saturable and obeying Michaelis-Menten kinetics, the second is apparently linear and indicates the value for the distribution ratio \( k_{e13}/k_{e31} \) of drug between erythrocytes and plasma.

**Figure 7.3b**
Relationship between plasma concentrations and red blood cell concentrations of chlorthalidone in 20 hypertensive patients who were receiving doses of 100 mg Hygroton®/day for 5 days or longer (closed points). The open circles represent data from human volunteers 24 hours after a single dose of chlorthalidone. Apparently the plot consists of two phases which can be analysed in the same way as was done in Figure 7.3a.
Linear approach

The compartment models employed in current pharmacokinetic literature are mathematical descriptions of the plasma, serum or blood decay curves, based upon a continuous first-order exchange between the central compartment (generally the sampling compartment, e.g. plasma) and the peripheral compartments (tissues) (Dost, 1953; Gibaldi and Perrier, 1975; Notari, 1975; van Rossum, 1971). The factors which can be used to relate the concentrations in the different compartments are distribution rate constants (e.g. hour⁻¹) or transfer clearance constants (e.g. liters/hour). In this way, hypothetical amounts of drug in tissues or tissue concentrations can be simulated, provided that the plasma concentrations are known. The use of the ratio of the transfer clearance constants of entry into and release from such compartment, \( k_{c,\text{in}} / k_{c,\text{out}} \), has the advantage of giving directly the ratio of the eventual tissue and plasma concentrations, provided that the state of equilibrium after absorption and distribution phases has been reached.

An implication of this description by linear differential equations is, that if the decay curves of plasma and tissue concentrations versus time are compared, they always appear to become parallel to each other, independently of how fast the equilibrium is reached (linear pharmacokinetics). This is shown in Figure 7.4 in which we brought about variations in the transfer rates of entry into and release from a peripheral compartment, and kept the ratio between them constant. The upper curves represent tissue compartments to which the drug is transferred preferentially compared with the central compartments. If the numerical value of \( k_{c,\text{out}} \) is lower it takes more time to reach the point where the tissue concentration is maximal. For those drugs which to an appreciable amount accumulate in the erythrocytes, the erythrocytes may be considered as a separate compartment, that belongs either to the central or the peripheral compartment depending upon the rate of drug exchange between plasma and erythrocytes.

Non-linear model

The results obtained with chlorthalidone are not consistent with description by means of a linear distribution model, in which the decay of plasma concentrations and red blood cell concentrations should become parallel soon. Therefore, we developed a model in which the erythrocytes were considered as a compartment composed of two parts: one portion of drug is exchangeable with the central compartment according to the appropriate distribution rate constants, the other portion is more tightly bound in a Michaelis-Menten type of binding, assuming a saturable number of bind-
Theoretical plasma concentration and tissue concentration curves on semi-logarithmic scale, based on a central and one tissue compartment and first-order absorption following oral administration of a drug with a considerable tissue uptake. Upper curves represent tissue concentrations, lower curves plasma concentrations. The parameters used in A, B, C are: D = 100 mg, $k_{ca} = 8 \text{ l/hr}$, $V_0 = 5 \text{ l}$, $V_1 = 40 \text{ l}$ (central), $V_2 = 2 \text{ l}$ (tissue) and $k_{cel} = 7.5 \text{ l/hr}$. The ratio between $k_{c12}$ and $k_{c21}$ was kept constant and the absolute magnitude of these transfer clearance constants varied as indicated in the figure parts A, B and C. In all cases the terminal decay curves of plasma and tissue concentrations become parallel, although this picture may be delayed initially by slow equilibration processes as is shown in Fig. 7.4C.

ing sites, expressed as that concentration of drug which can be bound maximally. The red blood cell concentrations measured experimentally are, of course, the sum of these two components.

In spite of the high concentrations found in the red blood cells with respect to the plasma (part of the central compartment) these two compartments could not be the only ones, as judged from the absolute amount of drug totally present there. The red blood cell concentration was known and the real red blood cell volume is a reasonably constant function of bodyweight (see e.g. Diem and Lentner, 1968). The plasma concentration is also known and the volume of the central compartment ($V_1$) can be estimated reliably from the time course of plasma concentrations. Corrections have to be made for the amount of drug not yet absorbed from the absorption compartment ($Q_0$) and the quantity already eliminated by the body ($Q_{el}$). The basic pharmacokinetic data from 8 experiments necessary for these calculations are summarized in Table 7.2. Details of the calculations are given in the Appendix 7.1. The resulting total amount, accounted for by the central compartment and red blood cells, was in every case smaller than the dose administered, for several values of bioavailability e.g. $F = 1$, $F = 0.5$ and $F = 0.25$. Therefore, it was concluded that another tissue compartment must be present over which chlorthalidone distributes and
### TABLE 7.2
Pharmacokinetic data after oral administration of chlorthalidone to human subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dose (mg)</th>
<th>$K_2$ (hr⁻¹)</th>
<th>$\alpha$ (hr⁻¹)</th>
<th>$\beta$ (hr⁻¹)</th>
<th>$V_1/F$ (liters)</th>
<th>$V_d/F$ (liters)</th>
<th>$k_{cat}/F$ (liters/hr)</th>
<th>AUC 0-10 hr (mg/liter/hr)</th>
<th>$C_1$ at $t = 10$ hr (mg/liter)</th>
<th>$C_2$ at $t = 10$ hr (mg/liter)</th>
<th>$V_3$ calc (liters)</th>
<th>$Q_2$ calc (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T S</td>
<td>100</td>
<td>0.862</td>
<td>0.392</td>
<td>0.0123</td>
<td>253</td>
<td>681</td>
<td>9.67</td>
<td>1.585</td>
<td>0.100</td>
<td>8.45</td>
<td>2.36</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.385</td>
<td>0.503</td>
<td>0.0171</td>
<td>320</td>
<td>828</td>
<td>15.8</td>
<td>2.42</td>
<td>0.165</td>
<td>11.4</td>
<td>2.36</td>
<td>41.1</td>
</tr>
<tr>
<td>D B</td>
<td>100</td>
<td>1.17</td>
<td>0.379</td>
<td>0.0139</td>
<td>214</td>
<td>708</td>
<td>12.1</td>
<td>1.634</td>
<td>0.093</td>
<td>9.00</td>
<td>2.32</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.195</td>
<td>0.361</td>
<td>0.0169</td>
<td>289</td>
<td>740</td>
<td>14.5</td>
<td>2.79</td>
<td>0.172</td>
<td>13.75</td>
<td>2.32</td>
<td>39.2</td>
</tr>
<tr>
<td>J B</td>
<td>100</td>
<td>0.694</td>
<td>0.415</td>
<td>0.0165</td>
<td>312</td>
<td>687</td>
<td>13.6</td>
<td>1.20</td>
<td>0.082</td>
<td>6.0</td>
<td>2.01</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.990</td>
<td>0.595</td>
<td>0.0193</td>
<td>352</td>
<td>806</td>
<td>17.8</td>
<td>1.86</td>
<td>0.132</td>
<td>7.20</td>
<td>2.01</td>
<td>53.4</td>
</tr>
<tr>
<td>M R</td>
<td>100</td>
<td>1.42</td>
<td>0.357</td>
<td>0.0165</td>
<td>200</td>
<td>500</td>
<td>8.0</td>
<td>1.735</td>
<td>0.152</td>
<td>6.70</td>
<td>1.98</td>
<td>42.5</td>
</tr>
<tr>
<td>T B</td>
<td>200</td>
<td>2.07</td>
<td>0.694</td>
<td>0.0199</td>
<td>214</td>
<td>728</td>
<td>17.0</td>
<td>2.33</td>
<td>0.150</td>
<td>7.90</td>
<td>1.85</td>
<td>56.8</td>
</tr>
</tbody>
</table>

*$K_a$, $\alpha$, and $\beta$ are the apparent first-order rate constants for absorption, first phase of disappearance, and terminal elimination, respectively, derived from the chlorthalidone plasma concentrations. The meaning of the other parameters shown is given in Appendix I.*
Figure 7.5
Schematic model used to explain the different elimination half-lives of the plasma and the red blood cell concentrations of chlorthalidone in man after oral administration. The absorption compartment has the subscript 0, volume \( V_0 \) and concentration \( Z \). The central compartment has the subscript 1, volume \( V_1 \) and concentration \( x \). The additional tissue compartment has the subscript 2, volume \( V_2 \) and concentration \( y \). The erythrocyte compartment has the subscript 3, volume \( V_3 \) and a concentration \( u + DE \) which is the sum of the concentration of exchangeable drug \( u \), and that of strongly bound drug \( DE \); \( u \) is in equilibrium with the concentration \( x \) in the central compartment according to the first-order transfer clearance constants \( k_{c13} \) and \( k_{c31} \).

where, relatively to plasma, high concentrations are reached. Expressed in percentages of the dose, the amount of drug calculated for this second tissue compartment \( Q_2 \) at 10 hours after oral administration, yielded mean values (n = 8) of \( Q_2 = 44.7\% \) (range 39-57\%) in the case of complete absorption from the gastrointestinal tract \( (F = 1) \), and for \( F = 0.5 \) and \( F = 0.25 \) respectively, mean values of \( Q_2 = 30.9\% \) (range 19-50\%) and \( Q_2 = 10\% \) (range 0-35\%).

The model eventually found appropriate for describing the pharmacokinetics of chlorthalidone is shown in Figure 7.5. The differential equations which are valid in this non-linear three compartment model are given in the Appendix 7.2. The influence of the binding capacity of the erythrocyte compartment on the elimination half-life from this compartment is shown in Figure 7.6. Three different situations were simulated, according to this model, by means of a computer program in which the binding capacity, expressed as \( B \) in Appendix 7.2, was simulated to be relatively low, intermediate and high with respect to the total chlorthalidone contents of the erythrocytes. It is clear that, whereas the plasma decay half-life varies only to a minor extent, changes in this binding parameter have a much greater influence on the elimination half-life from the erythrocytes.
Figure 7.6
Plasma and tissue concentration curves computed according to the three compartment model of drug distribution, in which the drug is tightly bound in the erythrocytes, giving rise to a prolonged elimination half-life compared with the plasma decay. Three situations are plotted in which the magnitude of the binding capacity B of the red blood cells is varied. It can be seen that the elimination half-life in the plasma compartment remains fairly constant, whereas that in the tissue compartment increases with increasing binding capacity. The model parameters used here were: $D = 100$ mg, $V_0 = 5$ l, $V_1 = 200$ l, $V_2 = 21$, $V_3 = 21$, $k_{c12} = 30$ l/h, $k_{c21} = 1$ l/h, $k_{c13} = 20$ l/h, $k_{c31} = 0.5$ l/h, $k_{ca} = 8$ l/h, $k_{cel} = 7.5$ l/h, $A = 1$ (mg/l)$^{-1}$, $B = 1$ (case a), $B = 5$ (case b), $B = 10$ (case c) (mg/l).

Figure 7.7 shows how the experimental data after a dose of 200 mg chlorthalidone to a healthy volunteer do agree with the model chosen, if appropriate parameters are set. The elimination half-life from the red blood cells is exceeding that from the plasma, while still the absorption into the red blood cells is reasonably fast. This picture would not be possible with the model shown in Figure 7.4, because in that situation, where apparently plasma decay is faster during the first period (e.g. Fig. 7.4c), it takes also more time to reach the maximum red blood cell concentration, which is not consistent with the experimental data.

From the distribution plots shown in Figures 7.3a and 7.3b a first ap-
proximation has been made for the parameters, describing the binding of chlorthalidone by the erythrocytes. These parameters were then used for curve-fitting according to the three compartment model shown in Figure 7.5. A consequence of the model chosen is that, in principle, the total binding of chlorthalidone to the red blood cells is not saturable because of the continuous distribution ratio between plasma and red blood cells, given by $k_{c13}$ and $k_{c31}$. The stronger binding sites, defined analogously to Michaelis-Menten kinetics by the maximum amount of drug bound $B$ and the affinity constant $A$, are saturable. The total concentration in the erythrocytes is a summation of these two binding phenomena as shown in Figure 7.3a. The saturable binding portion was obtained by stripping off
the line, which parallels the straight part of the curve and passes through the origin, from the original curve. The distribution ratio alone is given by the slope of this straight line.

In order to obtain a good fit (see Fig. 7.7) computer simulations were carried out at the beginning with estimated model parameters, until there was, judged by visual inspection, a reasonable agreement between the calculated curves and the experimental measurements. Finally, use was made of the computer program FARMFIT, a non-linear least squares regression analysis program (Chapter 1), which can employ also differential equations, in order to give model parameters which best fitted the data of the concentrations in plasma and erythrocytes simultaneously.

DISCUSSION

Experimental evidence is presented here for the influence of non-linear tissue binding on the pharmacokinetics of chlorthalidone in man, giving rise to a disposition rate from the erythrocytes which is slower than that from the blood plasma. There is good evidence that in this binding the enzyme carbonic anhydrase is involved (Beermann et al., 1975; Dieterle et al., 1976; This thesis, Chapter 8). In the model, the assumption is made that the time required for the uptake of the drug by the blood cell tissue in the Michaelis-Menten binding is very short compared with the transport of the drug to the tissue itself, governed by the distribution rate constants. This implies that at every moment a state of equilibrium between exchangeable drug and strongly bound drug in the erythrocytes is present. A similar theoretical model including tissue binding was given by Wagner (1971), but quantitative experimental measurements have not yet been described.

Just as the term \( x \) in the Appendix 7.2 refers to the total plasma concentration, which is in fact the sum of protein-bound and free drug, refers the term \( u \) to that concentration in the erythrocytes, which is the sum of really free drug and drug which is concerned in a binding of much lower affinity than the strong binding characterized by the Michaelis-Menten constants. Therefore, this concentration behaves as an exchangeable one, just as the plasma concentration, and the linear distribution between them can be described pharmacokinetically by the ratio of the transfer clearance constants \( k_{c13}/k_{c31} \). Because the ratio \( k_{c13}/k_{c31} \) is observed to be greater than unity, 'binding' is also involved in this linear distribution. But this binding (probably to proteins within the red blood cells with lesser affinity for the drug, but with greater capacity, as outlined in Chapter 8) shows up as a linear function of the plasma concentration (see Fig. 7.3). Because of the fact that the plasma protein binding of chlorthalidone was found to be
constant (75.7% bound) over the full concentration range studied, 0.02-7.7 μg/ml (Dieterle et al., 1976), no further possible non-linearity due to plasma protein binding has to be expected. The above implies that the exchangeable concentration u is directly proportional to the concentration of really free drug in the erythrocytes, which is, of course, the only species actually in equilibrium with the strongly bound drug. Therefore, this concentration u was used in the Michaelis-Menten relationship, shown in the Appendix 7.2.

Effects of binding phenomena on elimination half-lives of drugs are of course only visible if the maximum amount of drug which can be bound and the affinity constant for the binding places are in an order great enough to influence the half-life, e.g. see Figure 7.6. The parameter values for the binding capacity B and the distribution ratio $k_{c13}/k_{c31}$, found to fit adequately the experimental data in Figure 7.7, were at first estimated from the plots of plasma concentration vs. erythrocyte concentration (Figs. 7.3a and 7.3b), and then adjusted by refining the fits, as described in the pharmacokinetic analysis section of this chapter. It became clear that the apparent affinity constant for the binding of drug to the red blood cells, A in the Appendix, should be relatively low, as at higher values a bi-exponential decay of the erythrocyte concentrations with a slower phase would have been visible in the simulated plots, which was not justified by the experimental data.

Ultimately, however, after a long period of time, e.g. 200 hours or more, according to the model shown in Figure 7.5 and the differential equations in Appendix 7.2, also the plasma curve tended to accept a slower phase of elimination with the same disposition rate as the final red blood cell concentration decay. Observations, confirming this ultimate behaviour, will be dealt with in Chapter 9.

The solution presented in Fig. 7.7 is, however, by no means unique. This does not need to surprise, in view of the relatively great number of model parameters, as compared to the number of data points. Thus, strong mathematical correlation between parameter estimates results and the final solution depends heavily upon the adjustment of initial parameter values. Thus, an equally well fit to the experimental data of Fig. 7.7 was obtained e.g. with the model parameters: $D = 200$ mg, $V_0 = 101$, $V_1 = 3501$, $V_2 = V_3 = 41$, $k_{cel} = 15.6$ l/h, $k_{ca} = 20$ l/h, $k_{c12} = 92$ l/h, $k_{c21} = 11$ l/h, $k_{c13} = 32$ l/h, $k_{c31} = 0.68$ l/h, $A = 0.2$ (mg/l)$^{-1}$ and $B = 9$ mg/l. This indicates that prior to a definite choice of any set of parameters, additional data are needed. Such information could be provided by the accumulation pattern of plasma and red blood cell concentrations during repetitive dosing (see Chapter 13), and/or by a detailed study of the binding of chlorthalidone to red blood cells in vitro. The model parameters found until now show a rough agreement with the values calculated on the basis of the
known carbonic anhydrase content and the apparent affinity of chlorthalidone for carbonic anhydrase in the red blood cells, as will be described in Chapter 8.

In the computer fit shown to cover the experimental data in Figure 7.7 the parameters used did neglect incomplete absorption of the drug. In fact, uncertainty exists about the fraction of dose that was actually absorbed after oral administration of chlorthalidone. However, in the case that the bioavailability $F$ was not equal to unity, the same fits do apply to the data when the volume terms and clearance constants are multiplied by $F$. For instance, if $F$ is 0.5 instead of 1 exactly the same plot as shown in Figure 7.7 is generated with the parameter values $D = 100$ mg, $V_0 = 6$ l, $V_1 = 175$ l, $V_2 = 2$ l, $V_3 = 2$ l, $k_{ca} = 10$ l/h, $k_{cel} = 7.8$ l/h, $k_{c12} = 40$ l/h, $k_{c21} = 0.5$ l/h, $k_{c13} = 20$ l/h, $k_{c31} = 0.5$ l/h, $A = 1$ (mg/l)$^{-1}$ and $B = 6$ mg/l. These parameter values are probably closer to reality than those presented in Fig. 7.7, because of incomplete bioavailability of chlorthalidone: a mean value of $F = 0.64$ was found after oral administration of 50 mg doses, as is described in Chapter 9.

The first reason for the choice of an additional tissue compartment in the model was evident from the consideration of the total amount of drug accounted for by the central compartment and the red blood cells, which aspect was elucidated in the pharmacokinetic analysis of this Chapter. An additional argument for the use of three compartments was given by the practice of the fitting procedure: the observed rapid decay of plasma concentrations in the initial period (between 3 and 10 hours after the dose) could be simulated with a high numerical value for the clearance constant $k_{c13}$. However, the choice of this $k_{c13}$ is limited in its upper value by the measured entrance rate of drug into the erythrocytes. In order to reconcile these observed features a second way of disappearance from the central compartment had to be included ($k_{c12}$). With respect to the diuretic action of the compound it is well possible that renal tissue will be a major portion of this compartment.

With respect to the great central volume of distribution $V_1$ found to fit the measured data, it is clear that this volume cannot represent a single uniform volume, but consists, besides of the plasma volume, of a compartment which equilibrates with the blood plasma very rapidly relative to the rate of absorption of the drug. After oral administration its presence is not visible as an additional distribution phase in the plasma concentration curve. The investigations on the intravenous administration of chlorthalidone to human subjects, described in Chapter 9, revealed the existence of such a rapid phase of decay, with a mean half-life of ca. 20 min. Consequently, in that study smaller values for the volume of the central compartment than those found after oral administration resulted. For reasons of simplicity, however, this compartment was not shown explicitly in the
model (Fig. 7.5), but was included in the central compartment. In rat experiments chlorthalidone showed a considerable liver uptake immediately after administration, followed by excretion into the bile (Beisenherz et al., 1966). In view of this, the liver could be considered very well as an important part of the central volume of distribution for this drug.

CONCLUSION

The non-linear pharmacokinetic model, shown in Figure 7.5, is able to include simultaneously all observed features of the time course of absorption, distribution and elimination of chlorthalidone in both the plasma and erythrocyte compartment. Numerical solutions obtained are not unique, however, due to a small number of degrees of freedom. It should be noticed in addition, that the model was constructed in the most simple way, by which a satisfying fit to the experimental data could be obtained. The binding of chlorthalidone to red blood cells, for instance, was composed of only two parts. This seemed justified, judged from the pattern of distribution of the drug between plasma and red blood cells (Fig. 7.3). However, as explained in Chapter 8, three components are actually found in red blood cell binding of chlorthalidone, if a more extended concentration range is employed. If this factor would be introduced, a still more complex non-linear model would arise, from which naturally different parameter estimates, e.g. transfer clearance constants, would be found. Concludingly, these circumstances cause severe limitations to the applicability of the non-linear model, unless its individual elements would be precisely specified by additional experimental data. For this reason, the model proposed will be applied merely, in the following chapters of this thesis, to explain observations, which are clearly conflicting with a linear pharmacokinetic concept. E.g. in Chapter 13 the capacity-limited binding of chlorthalidone to red blood cells will be shown to have a pronounced influence on its pharmacokinetics during multiple dosing. Details of the absorption and elimination phases of separate plasma and red blood cell concentration curves of chlorthalidone can, for the moment, with less uncertainty in parameter estimates, better be described by linear differential equations, as used in Chapter 9. It should be realized, however, that such a description, although valuable of course for comparison of pharmacokinetic parameters of chlorthalidone with those reported for other drugs, causes a lesser degree of understanding about the coherence and physiological meaning of the various compartments in the body. No doubt, therefore, integration of all available individual pieces into one dynamic system will ultimately lead to the best possible explanation of what such 'compartments' really represent.
SUMMARY

Plasma concentrations and red blood cell concentrations of chlorthalidone have been measured after oral administration of single doses of 100 or 200 mg to ten human subjects. Chlorthalidone was rapidly absorbed into plasma, with peak concentrations occurring at 1-3 hours after drug intake. The transport to erythrocytes was much slower and maximum concentrations were not reached until 10-15 hours later. The concentration of chlorthalidone in erythrocytes was then 50-100 times higher than that in plasma. The decay of red blood cell concentrations showed a much longer elimination half-life (mean 60 hours) than the terminal plasma decay (mean 40 hours). The in vitro distribution of chlorthalidone between plasma and erythrocytes was non-linear and similar to the in vivo distribution curve obtained from patients who were in a steady-state concentration range.

A pharmacokinetic model was developed, including non-linear binding of chlorthalidone by the red blood cells, which in detail could account for the observed time courses of drug in plasma and erythrocytes simultaneously. Applicability and limitations of this model were discussed and compared with linear pharmacokinetic analysis.

APPENDIX 7.1

The amount of drug totally present in all body compartments plus the quantity eliminated by the body equals the dose at every time t:

\[ Q_0(t) + Q_1(t) + Q_2(t) + Q_3(t) + Q_{el}(t) = D \]

where:

- \( Q_0(t) \) = the dose of drug
- \( Q_1(t) \) = the quantity of drug (e.g., mg) in the absorption compartment at time t
- \( Q_2(t) \) = the quantity of drug (e.g., mg) in a possibly existing additional tissue compartment at time t
- \( Q_3(t) \) = the quantity of drug (e.g., mg) in the red blood cells at time t
- \( Q_{el}(t) \) = the quantity of drug eliminated by the body at time t

\( C_1(t) \) and \( V_1 \) are, respectively, concentration at time t and volume of the central compartment.

\( C_3(t) \) and \( V_3 \) are, respectively, concentration at time t and volume of the red blood cell compartment.
\[ Q_{el}(t) = \text{the quantity of drug (e.g., mg) already eliminated from the body at time } t. \] 
If the elimination occurs from the central compartment:

\[
Q_{el}(t) = k_{cel} \int_0^t c_1 \, dt = k_{cel} \cdot \text{AUC}
\]

where \( k_{cel} \) is the clearance constant of elimination (e.g., l/h) and \( \text{AUC} \) is the area under the plasma concentration vs time curve (e.g., mg.hr/l)

\( Q_2(t) \) can be calculated if all other quantities are known. As the plasma concentration curves showed a biphasic decay, these curves were fitted to a three-exponential equation according to a linear two-compartment model, with first-order drug distribution and elimination after oral administration, as described in Chapter 9. The resulting pharmacokinetic data are given in Table 7.2. This approximation yields values for \( V_1/F \), the total apparent volume of distribution \( V_{dss}/F \), the rate constant of absorption \( K_a \) and the clearance constant of elimination \( k_{cel}/F \), which is related with the elimination rate constant by \( k_{el} = k_{cel} \).

Because of the fact that the fraction of dose absorbed from the gastrointestinal tract (bioavailability \( F \)) was not actually known, the values for \( V_1/F, V_{dss}/F \) and \( k_{cel}/F \), shown in Table 7.2, have to be multiplied by \( F \). Cumulative urinary excretion of chlorthalidone measured during 4 days in the subjects studied ranged between 25 and 50\% of the dose, which indicates a minimum value for \( F \). Estimates of \( V_1 \) and \( k_{cel} \), necessary for the calculation of \( Q_1 \) and \( Q_{el} \) respectively, where thus obtained using different values of bioavailability: \( F = 1, F = 0.5 \) and \( F = 0.25 \).

As judged from the values for \( K_a \) (Table 7.2), absorption is fast and after 10 hours only a negligible fraction of the available dose will remain in the absorption compartment. Therefore, \( Q_0 \) was set at zero at this time and the calculations were carried out at \( t = 10 \) h. \( C_1(t) \) and \( C_3(t) \) were measured experimentally and are shown in Table 7.2 for \( t = 10 \) h. \( V_3 \) is a function of bodyweight, the red blood cell volume being equal to about two liters for a 70 kg man. The area under the curve (AUC) from \( t = 0 \) until \( t = 10 \) h was obtained with the trapezoidal rule and multiplied subsequently with \( k_{cel} \) to yield \( Q_{el} \) at \( t = 10 \) h. Finally, \( Q_2 \) was obtained for each of the three values of \( F \) mentioned by subtracting the other quantities from the available dose F.D. The results of these calculations are presented in the last three columns of Table 7.2.
APPENDIX 7.2

In the model depicted in Figure 7.5 the following differential equations are valid:

\[ \frac{dQ_0}{dt} = -k_{ca} \cdot Z \quad \text{where} \quad Q_0 = V_0 \cdot Z \quad \text{(Eq.7.1)} \]

\[ \frac{dQ_1}{dt} = k_{ca} \cdot Z - (k_{cel} + k_{c12} + k_{c13}) \cdot x + k_{c21} \cdot y + k_{c31} \cdot u \]
\[ \quad \text{where} \quad Q_1 = V_1 \cdot x \quad \text{(Eq.7.2)} \]

\[ \frac{dQ_2}{dt} = k_{c12} \cdot x - k_{c21} \cdot y \quad \text{where} \quad Q_2 = V_2 \cdot y \quad \text{(Eq.7.3)} \]

\[ \frac{dQ_3,u}{dt} = k_{c13} \cdot x - k_{c31} \cdot u - V_3 \cdot \frac{d \cdot DE}{dt} \]
\[ \quad \text{where} \quad Q_3,u = V_3 \cdot u \quad \text{(Eq.7.4)} \]

\[ \frac{dQ_{el}}{dt} = k_{cel} \cdot x \quad \text{(Eq.7.5)} \]

The boundary conditions are:

at \ t = 0: \ Z = D/V_0 \quad x = y = u = 0 \quad Q_{el} = 0

at \ t = \infty: \ Z = 0 \quad x = 0 \quad y = 0 \quad u = 0 \quad Q_{el} = D

The concentration \ DE \ is related to \ u \ according to Michaelis-Menten kinetics by means of the relationship

\[ DE = \frac{u \cdot A \cdot B}{1 + A \cdot u} \quad \text{(Eq.7.6)} \]

where \ A \ represents the apparent association constant of the drug-erythrocyte binding and \ B \ the maximum amount of binding sites, expressed as that concentration of drug that can be bound maximally.
The differential equation 7.4 can be transformed with the relationships

\[ \frac{dQ_3}{dt}, u = \frac{du}{dt} \quad \text{and} \quad \frac{d\text{DE}}{dt} = \frac{d\text{DE}}{du} \cdot \frac{du}{dt} \]

to

\[ \frac{dQ_3}{dt}, u = \frac{k_{c13} \cdot x - k_{c31} \cdot u}{1 + \frac{d\text{DE}}{du}} = \frac{k_{c13} \cdot x - k_{c31} \cdot u}{1 + \frac{A \cdot B}{(1+A \cdot u)^2}} \quad \text{(Eq. 7.7)} \]

As far as we know, there is no analytical solution for this set of differential equations but the equations 7.1, 7.2, 7.3, 7.5 and 7.7 can be easily solved numerically by computer to yield simulations of Z, x, y and u as a function of time. DE as function of u, see Eq. 7.6, is therefore also known as well as the sum u + DE, which was actually measured experimentally.

A glossary of terms, not yet explained in the caption of Figure 7.5 follows:

\[ \frac{dQ_{\text{el}}}{dt} = \text{the quantity of drug (e.g., mg) eliminated per unit of time (e.g., hr)} \]

\[ k_{\text{cel}} = \text{the clearance constant of elimination from the central compartment (e.g., liters/hr) assuming linear elimination kinetics} \]

\[ D = \text{the dose of the drug (e.g., mg)} \]

\[ Q_0 = \text{the quantity of drug (e.g., mg) in the absorption compartment} \]

\[ Q_1 = \text{the quantity of drug (e.g., mg) in the central compartment} \]

\[ Q_2 = \text{the quantity of drug (e.g., mg) in the second tissue compartment} \]

\[ Q_3, u = \text{the quantity of drug (e.g., mg) in that part of the erythrocyte compartment, which contains the exchangeable drug concentration } u \]

\[ k_{\text{ca}} = \text{the clearance constant of disappearance from the absorption compartment to the central compartment (e.g., liters/hr); } k_{\text{ca}} \text{ is directly related to the rate constant of drug absorption } K_a \text{ (e.g., hr}\text{)}^{-1} \text{ by } k_{\text{ca}}/V_0 = K_a \]

\[ k_{c12} = \text{the transfer clearance constant of disappearance from the central compartment to the compartment with number 2 (e.g., liters/hr); } k_{c12} \text{ is directly related to the rate constant } K_{12} \text{(e.g., hr}^{-1}) \text{ by } k_{c12}/V_1 = K_{12}. \text{ The transfer clearance constants } k_{c21}, k_{c13}, k_{c31} \text{ are defined analogously, so that } k_{c21}/V_2 = k_{21}; k_{c13}/V_1 = k_{13}; k_{c31}/V_3 = k_{31}. \]
REFERENCES

Beermann, B, Hellström, K, Lindström, B, Rosen, A Binding-site interaction of chlor thalidone and acetazolamide, two drugs transported by red blood cells *Clin Pharmacol Ther* 17, 424-432 (1975)

Beisenherz, G, Koss, F W, Klatt, L, Binder, B Distribution of radio-activity in the tissues and excretory products of rats and rabbits following administration of C^{14}-Hygroton *Arch int Pharmacodyn* 161, 76-93 (1966)


Dieterle, D, Wagner, J, Faigle, J W Binding of chlorthalidone (Hygroton®) to blood components in man *Europ J clin Pharmacol* 10, 37-42 (1976)

Dost, F H, *Der Blutspiegel* Leipzig Thieme 1953

Fleuren, H L J, van Rossum, J M Pharmacokinetics of chlorthalidone in man *Pharm Weekbl* 110, 1262-1264 (1975)


Gibaldi, M, Perrier, D *Pharmacokinetics* New York Marcel Dekker 1975

Mach, R S, Veyrat, R Clinical experiences with some of the newer diuretics, especially chlorthalidone *Ann N Y Acad Sci* 88, 841 863 (1960)

Notari, R E *Biopharmaceutics and Pharmacokinetics, 2nd ed* New York Marcel Dekker 1975


Wagner, J G *Biopharmaceutics and Relevant Pharmacokinetics*, pp 302-317 Hamilton, Ill Drug Intelligence Publications 1971
INTRODUCTION

After administration of chlorthalidone to human subjects, the concentration of the drug in red blood cells is 50-100 times higher than that in plasma, as is described in Chapter 7. There is sufficient evidence now to state that the intracellular protein carbonic anhydrase is involved in this uptake. The drug acts as a weak inhibitor of this enzyme in vitro (Pulver et al., 1962; Maren, 1967). Acetazolamide, a stronger inhibitor than chlorthalidone, is most probably bound to the red cell enzyme (Coleman, 1975; Maren et al., 1960; Wallace and Riegelman, 1977), and displaces chlorthalidone from its erythrocyte binding sites in vivo and in vitro (Beermann et al., 1975). Gel chromatographic separation of red cell constituents revealed that chlorthalidone eluted from the column only at the retention time of the carbonic anhydrase fraction (Beermann et al., 1975; Dieterle et al., 1976).

The mean concentration of total carbonic anhydrase in human erythrocytes is 140-160 µmol/liter (Maren, 1967; Wistrand and Baathe, 1968; Nørgaard-Pedersen and Mondrup, 1971), but individual values may vary from 120-225 µmol/liter (Nørgaard-Pedersen and Mondrup, 1971). In agreement with saturation of this pool of receptors, the red blood cell binding curve of chlorthalidone shows a clear inflexion point in the same concentration range, as is shown in Fig. 8.1 (Experiment performed as described in the Materials and Methods section; a similar observation has been made by Dieterle et al., 1976). Dieterle et al. (1976) determined the affinity of the drug for two isolated isoenzymes of human carbonic anhydrase (HCA), so called HCA-B and HCA-C. They reported one binding site per molecule of either isoenzyme and a $K_{ass} = 2.4 \times 10^6$ 1/mole for HCA-B and a $K_{ass} = 5.7 \times 10^6$ 1/mole for HCA-C. The association constants were several orders of magnitude larger than that with respect to human serum albumin ($K_{ass} = 1.2 \times 10^3$ 1/mole), and this difference was in agreement with the large erythrocyte over plasma concentration ratios of chlorthalidone observed in vivo.

A discrepancy became apparent, however, between details of the report of Dieterle et al. (1976). Only one component of binding was discerned
Figure 8.1
Graphs showing the concentration of chlorthalidone in plasma and red blood cells after equilibration of the drug in whole blood from a normal human subject. The erythrocyte over plasma concentration ratio (left part) goes down from about 80 to 1.5 when the whole blood concentration is increased from 0.3 to 240 μg/ml. The right panel presents the concentration in erythrocytes as a function of plasma concentration. The saturable part of the binding corresponds to a red blood cell concentration of chlorthalidone of 68 μg/ml (201 μM) in this experiment, which is in the normal range of the total carbonic anhydrase concentration in human erythrocytes.

when the mixture of HBC-B and HCA-C from erythrocytes was employed. This is clearly surprising because of the difference in the affinity of chlorthalidone for HCA-B and HCA-C and in view of the relative occurrence of the two isoenzymes. The quantity of HCA-C is about 6-8 times smaller than that of HCA-B, which means that its erythrocyte concentration lays between 15-25 μM (Nørgaard-Pedersen and Mondrup, 1971), a certainly significant amount. It must be expected therefore that not the total carbonic anhydrase pool but merely the isoenzyme with the highest affinity acts as the primarily saturable receptor of chlorthalidone in red blood cells. Some evidence for this hypothesis can be found in Chapter 7 (Fleuren and van Rossum, 1977), showing a small saturable component of binding in the
in vivo concentration range (cf. Fig. 7.3: \( B = 6.8 \mu g/ml \), equivalent to 20 \( \mu M \)). Furthermore, it should be realized that the very high drug concentrations employed in the in vitro experiment of Fig. 8.1 are never encountered in man after administration of single doses, where peak concentrations of about 10-15 \( \mu g/ml \) are attained in red blood cells and of less than 1 \( \mu g/ml \) in plasma (cf. Chapter 7).

Because many kinetic events take place simultaneously in vivo, single processes are difficult to isolate. This difficulty can be circumvented by means of a closed system. An in vitro study with intact blood corpuscles was expected to approximate the in vivo situation more closely than experiments with isolated proteins would do, because all components of binding, also those of low affinity, would be taken into account. Moreover, such study would provide also information on the rate of transport of chlorthalidone across the cell membrane. The major aim of this chapter was, however, to find out whether in the concentration range corresponding to the total amount of red cell carbonic anhydrase the two components of specific binding anticipated for chlorthalidone could really be distinguished.

MATERIALS AND METHODS

**Drug partitioning studies**

Chlorthalidone was obtained from Ciba-Geigy (Basle, Switzerland). Its purity was ascertained by thin-layer chromatography as described in Chapter 3. All inorganic chemicals were of analytical reagent grade (E. Merck, Darmstadt, G.F.R.). The heparin solution contained 50 mg (5000 U) per ml (Thromboliquine, Organon, Oss, The Netherlands). Blood portions of 50-100 ml with hematocrit 0.40-0.50 were collected by venepuncture from healthy human subjects, who had fasted overnight. Care was taken to avoid hemolysis, viz. by using a wide-bore needle and by a cautious heparinization of the blood (see e.g. Hanks et al., 1960), 0.2 ml of heparin solution being used for 100 ml of whole blood. (Heparin does not influence the distribution of chlorthalidone between plasma and erythrocytes, as reported in Chapter 3). The blood was used the same day or the day thereafter for incubations with the drug. These were carried out in glass flasks by regular and gentle agitation at 20°C or 37°C. Previously, it had been checked that the drug was stable in whole blood or in suspensions of red cells in buffer during at least 48 hours.

The time course of equilibration of chlorthalidone between plasma (or physiological saline) and erythrocytes was studied by dissolving the drug in a small volume of 0.1 M NaOH containing 0.3% (w/v) of sodium chloride
and adding this to whole blood (0.1 ml on 5 ml of blood) at varying drug concentration (0.5-10 μg/ml). Next, separation of plasma (or physiological saline) and red cells was carried out as described in Chapter 3 at several times after the start of the incubation, see Fig. 8.2 and Table 8.1. The erythrocytes were assayed for chlorthalidone directly and after being washed once or twice with 0.9% (w/v) NaCl. Analogously, the rate of transport of chlorthalidone out of the erythrocytes was measured after suspending red blood cells, which had been pre-incubated with the drug, in fresh portions of physiological saline.

To study the equilibrium red cell over plasma distribution in whole blood, the drug was pre-dissolved in plasma (350 μg/ml) and aliquots of this solution were added to whole blood from which an equal volume of plasma had been removed before, so that the hematocrit remained unchanged. The highest blood concentrations of the range 0.3-240 μg/ml (Fig. 8.1) could only be achieved by repeated replacement of incubated plasma by a new aliquot of the stock plasma solution. A 120 min period was used to reach equilibrium distribution.

Red blood cell suspensions were prepared in an isotonic phosphate buffer consisting of Na₂HPO₄.2H₂O 3.674 g/l, KH₂PO₄ 0.684 g/l and NaCl 7.78 g/l. The osmolarity of this buffer was 305 mOsm/l and its pH 7.43 at 20°C. After collection of the blood, the plasma was separated by centrifugation at 3000 r.p.m. (1500 g) for 15 min, the erythrocytes were washed three times with four times their volume of the isotonic phosphate buffer, and diluted with this buffer to hematocrit 0.50. Precise 1 ml portions of this suspension were added to twenty 4 ml portions of isotonic buffer, containing each a known amount of chlorthalidone between 0.5 and 250 μg (1.47-738 nmole). After incubation for 3 hours at 37°C, the samples were centrifuged and the supernatant fluid was assayed. Erythrocyte concentrations were calculated from the total amounts added, the concentrations in the buffer solution and the hematocrit of the cell suspension.

The hematocrit was measured in triplicate by means of a Hawskley micro-hematocrit centrifuge (Hawskley, Lancing, England), and the readings were corrected for a fluid inclusion percentage of 3% (England et al., 1972). The concentration of chlorthalidone in plasma, red cells or buffer was determined by gas chromatography with nitrogen detection (Fleuren and van Rossum, 1978; Chapter 3).

Analysis of erythrocyte-binding sites

At equilibrium, the concentration of unbound drug within the cells is assumed to be equal to the concentration of drug in the buffer solution outside. The total concentration of chlorthalidone in the cells (RX) can then be written as a function of the concentration of unbound drug (X) in the surrounding buffer:
In this modified Scatchard equation (Scatchard, 1949) \( n \) is the number of classes of binding-sites assumed to be independent of each other, \( N_i \) is the maximum binding capacity of each class, which is the product of the concentration of the binding macromolecule and the number of sites on that macromolecule, and \( K_i \) the apparent association constant for each class. By means of the term \( k_3X \) several processes are lumped together. If no other components of binding than those already characterized by a \( N_i \) and a \( K_i \) ("specific binding sites") are present, then \( k_3 = 1 \). If, however, also low affinity binding sites are involved of which no saturation is apparent (i.e., \( X \) is far below the dissociation constants corresponding to those sites), such "non-specific binding" shows up as a linear function of the concentration of unbound drug and \( k_3 \) is larger than one.

Preliminary graphical estimation of the binding parameters according to the method of Rosenthal (1967) showed that either one or two specific binding sites were present. Both possibilities were explored by fitting the experimental data to Equation 8.1 by use of a non-linear least squares regression analysis program (Farmfit, see Chapter 1), with weights equal to \( 1/(RX)^2 \). This computer program provided also a measure for the asymptotic standard error in the parameter estimates. To decide between \( n = 1 \) and \( n = 2 \), several criteria were used: 1. Visual inspection of the Scatchard plots for goodness of fit. 2. The distribution of the residuals around the fitted curves should be as much as possible at random. 3. A statistical comparison of the goodness of fit by means of the \( f \)-test (see e.g. Boxenbaum, 1974; cf. Chapter 9). This test indicates if the reduction in the total sum of weighed squared deviations of the data points gained by use of the more complicate equation \( (n = 2 \text{ instead of } n = 1) \) is statistically significant at a certain probability level, e.g. \( P = 0.01 \).

RESULTS

Rate of distribution of chlorthalidone between plasma and erythrocytes in vitro

Chlorthalidone did not equilibrate instantaneously between plasma (or buffer) and erythrocytes. In fresh human blood or blood cell suspensions, a constant concentration ratio was attained at 37°C after 90-120 minutes. This held true both for uptake of the drug by and release from
Figure 8.2
Typical plots showing first-order rate of transport of chlorthalidone from plasma to red blood cells at two temperatures. At 37°C equilibrium is reached after 90-120 minutes. At 20°C the transport rate is much slower.

erythrocytes. At 20°C equilibrium was not reached even after 4 hours. The transport rate occurred in a first-order fashion during the first part of the incubation period, see Fig. 8.2. Three experiments with blood of normal composition yielded a transport half-life of 11 ± 2 min at 37°C (mean value ± S.D.).

Washing the erythrocytes with physiological saline had a negligible effect upon the chlorthalidone concentration. This was the case both when the concentrations of the drug in plasma and erythrocytes were in equilibrium and during the transport of chlorthalidone from the plasma to the cells (Table 8.1).

Red blood cell binding parameters
The results of a typical experiment are presented as a Scatchard plot in Fig. 8.3. When two classes of specific binding were taken into account a much better fit was obtained than that based upon one class only. This visual judgement was supported by the way the residuals of the data points were distributed around the fitted curves and by the outcome of the f-test. This test indicated for all six experiments a very significant improvement (P < 0.005), if five instead of three binding parameters were employed.
### TABLE 8.1
Effect of washing of red blood cells upon erythrocyte concentrations of chlorthalidone

<table>
<thead>
<tr>
<th>Incubation time&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>Plasma conc. (μg/ml)</th>
<th>Red blood cell concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.7</td>
<td>unwashed</td>
</tr>
<tr>
<td>1</td>
<td>5.9</td>
<td>washed (2 x)</td>
</tr>
<tr>
<td>5</td>
<td>3.9</td>
<td>unwashed</td>
</tr>
<tr>
<td>10</td>
<td>2.2</td>
<td>washed (2 x)</td>
</tr>
<tr>
<td>15</td>
<td>1.35</td>
<td>unwashed</td>
</tr>
<tr>
<td>30</td>
<td>0.39</td>
<td>washed (2 x)</td>
</tr>
<tr>
<td>90</td>
<td>0.17</td>
<td>unwashed</td>
</tr>
<tr>
<td>150</td>
<td>0.17</td>
<td>washed (2 x)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Experiment performed at 37°C

### TABLE 8.2
Parameters characterizing the binding of chlorthalidone to human erythrocytes in vitro<sup>a</sup>

<table>
<thead>
<tr>
<th>Exp.&lt;sup&gt;b&lt;/sup&gt; no.</th>
<th>K&lt;sub&gt;1&lt;/sub&gt; (x10^-6 M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>N&lt;sub&gt;1&lt;/sub&gt; (μM)</th>
<th>K&lt;sub&gt;2&lt;/sub&gt; (x10^-6 M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>N&lt;sub&gt;2&lt;/sub&gt; (μM)</th>
<th>k&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1.08 (15)</td>
<td>151 (4)</td>
<td>21.9 (43)</td>
<td>9.6 (42)</td>
<td>3.90 (5)</td>
</tr>
<tr>
<td>1B</td>
<td>0.96 (36)</td>
<td>136 (15)</td>
<td>6.8 (54)</td>
<td>34.2 (73)</td>
<td>2.97 (8)</td>
</tr>
<tr>
<td>2</td>
<td>0.92 (8)</td>
<td>137 (4)</td>
<td>73.5 (43)</td>
<td>3.8 (24)</td>
<td>3.22 (5)</td>
</tr>
<tr>
<td>3</td>
<td>0.85 (7)</td>
<td>204 (3)</td>
<td>45.2 (32)</td>
<td>5.3 (25)</td>
<td>2.24 (9)</td>
</tr>
<tr>
<td>4</td>
<td>1.00 (22)</td>
<td>146 (6)</td>
<td>10.4 (74)</td>
<td>13.0 (82)</td>
<td>3.38 (7)</td>
</tr>
<tr>
<td>5</td>
<td>1.06 (12)</td>
<td>156 (4)</td>
<td>19.9 (44)</td>
<td>7.6 (47)</td>
<td>3.71 (4)</td>
</tr>
</tbody>
</table>

<sup>a</sup>K<sub>1</sub> and K<sub>2</sub> are apparent association constants, and N<sub>1</sub> and N<sub>2</sub> maximum binding capacities; k<sub>3</sub> is the linear distribution parameter (relative errors of the parameter estimates in parentheses).

<sup>b</sup>Numbers indicate experiments performed with red blood cell portions from different subjects.
Figure 8.3
Scatchard plot analysis of red blood cell uptake of chlorthalidone (exp. no. 3) indicates the presence of two classes of binding sites with high affinity for the drug, and an additional uptake of low affinity. The inset represents the line of best fit if only one class of saturable binding sites was taken into account. The picture has been reproduced directly from a computer plot.

Table 8.2 presents the parameter estimates obtained by fitting the experimental data according to Equation 8.1 with \( n = 2 \).

DISCUSSION

The observation of a transport process with an initial first-order rate (Fig. 8.2) can easily be explained from a fast association reaction of chlorthalidone with intracellular carbonic anhydrase. This binding will keep the free drug concentration low, so that the driving force for transport - the concentration gradient between the outside and the inside of the erythrocytes - is maintained. Also the results shown in Table 8.1 indicate that diffusion across the cell membrane and not formation of the drug-protein complex is the rate-limiting step in the red cell uptake of chlorthalidone. Would the association reaction itself have been a slow process, and the diffusion rate fast, then washing of the red cells would have re-
moved the unbound drug fraction and would have resulted in smaller concentrations. The rapid establishment of the binding of chlorthalidone to carbonic anhydrase would be in agreement with the fast protein-complex formation of 24 other sulfonamides, including acetazolamide, for which association rate constants ranging from approximately $10^5-10^7$ M$^{-1}$ sec$^{-1}$ have been measured (Coleman, 1975).

During our preliminary experiments, it was noticed that the rate of distribution of chlorthalidone between plasma and red blood cells depended also to some extent upon the age of the blood. In whole human blood kept for a few days in a refrigerator a constant distribution ratio was reached after 60 min (cf. Fig. 3.5, Chapter 3), while it took 90-120 min when blood was incubated with the drug the day of collection itself (see e.g. Fig. 8.2). This circumstance may be related to the slow equilibration of chlorthalidone between plasma and red blood cells in vivo: even after intravenous administration of the drug maximum red blood cell concentrations are not found before 6-9 hours (Fleuren et al., 1979; Chapter 9). Such a difference between the in vivo and in vitro rate of distribution in blood was noticed also in the rat (Beisenherz et al., 1966). The reason for this discrepancy is not clear, but it may be supposed that an alteration of the red cell membrane occurs outside the body, or that some factor acting in vivo, e.g. a substance competing for transport, is absent or lost in vitro.

Scatchard plot analysis revealed the existence of two saturable classes of binding for chlorthalidone in the concentration range encountered after single doses (Fig. 8.3, Table 8.2). The molar quantity of the largest of these components, $136-204 \mu$M in six experiments is in good agreement with the known red cell content of one of the isoenzymes of human carbonic anhydrase, HCA-B, which has been reported to range from 115-220 $\mu$M (Nørgaard-Pedersen and Mondrup, 1971). Also the apparent association constant, $1 \times 10^6$ 1/mole on the average, is near the figure reported by Dieterle et al. (1976) on the affinity of chlorthalidone for isolated HCA-B ($K_{ass} = 2.4 \times 10^6$ 1/mole). A second possibility, however, which cannot entirely be excluded is that this quantity corresponds to the concentration of total carbonic anhydrase in human erythrocytes, which amounts to 130-230 $\mu$M (Nørgaard-Pedersen and Mondrup, 1971). In this case, binding to the isoenzyme HCA-C would apparently be indistinguishable from that to HCA-B, and the binding sites characterized by $K_2$ and $N_2$ in Table 8.2 would belong to a hitherto unidentified receptor. Such a hypothesis, however, is not supported by studies employing column chromatography of hemolysed red blood cells, which demonstrated no other site of accumulation for chlorthalidone than that corresponding to the combined carbonic anhydrase fraction (Beermann et al., 1975; Dieterle et al., 1976).

In view of the strong mathematical correlation of the parameters $K_2$ and $N_2$, the only justified conclusion can be that the data shown in Table 8.2 do
not yet allow a definite statement with respect to this point, and that ex­periments with more data points in the very low concentration range are needed to reduce the large errors of the estimates of $K_2$ and $N_2$. This is il­lustrated also by the difference between the estimates for $K_2$ and $N_2$ ob­tained from two experiments with the same blood portion, performed one day apart (Experiments 1A and 1B in Table 8.2).

SUMMARY

The binding of chlorthalidone to human red blood cells was studied in vitro. The initial rate of erythrocyte uptake of the drug from plasma or buffer was first-order (with a half-life of ca. 10 min at $37^\circ C$), which was consist­ent with a fast establishment of intracellular binding. Approximately 90-120 minutes were needed to reach full equilibrium between the concentrations in plasma (or buffer) and erythrocytes. The total binding of chlorthalidone to red blood cells appeared to consist of three components: two classes of binding characterized by apparent association constants and maximum binding capacities, and a non-specific type of binding, showing up as a linear function of the unbound drug concentration. The first class of saturable binding sites had a maximum binding capacity of 155 $\mu$M on the average (six experiments, range 136-204 $\mu$M), and the apparent association constant for binding of chlorthalidone was $1 \times 10^6$ 1/mole (range 0.9-1.1$ \times 10^6$ 1/mole). These data were in good agreement with literature data on the molar content of and affinity of chlorthalidone for the B-isoenzyme of red cell carbonic anhydrase. The second class of binding sites could be defined less precisely: the estimates of the apparent association constant of the drug-protein complex ranged from 7-74 $\times 10^6$ 1/mole and the maximum binding capacity from 4-34 $\mu$M. Due to the large uncer­tainty in these parameter estimates, assessment of the C-isoenzyme of human carbonic anhydrase as the binding protein anticipated to be invol­ved here should be regarded therefore as preliminary.

REFERENCES


**ERRATUM WITH RESPECT TO CHAPTER 9:**

In stead of page 177 page 184 should be read.

In stead of page 184 page 177 should be read.

In stead of page 185 page 192 should be read.

In stead of page 192 page 185 should be read.
INTRODUCTION

In the Chapters 7 and 8, the distribution of chlorthalidone between plasma and erythrocytes was shown to be concentration-dependent. The non-linear model, which included besides first-order processes also a capacity-limited binding of chlorthalidone to red blood cells, enabled explanation of the longer half-life of chlorthalidone concentration in erythrocytes compared to that in plasma. However, as described in the Discussion section of Chapter 7, no unique solution for the parameters of this model could be obtained. Therefore, we considered it more practical for the purpose of the present bioavailability study to describe plasma and red blood cell concentration decay separately, according to conventional linear open compartment models, of which a general description has been given in Chapter 1. Preliminary curve-fitting of the experimental data indicated that plasma concentrations of chlorthalidone, as long as they could be followed by means of our assay, could be described in this way, implying that rate constants (or alternatively, time constants) of absorption, distribution and elimination would be obtained with good precision. Erythrocyte concentrations of chlorthalidone appeared to rise in a first-order manner and, as the decline thereafter was mono-exponential (see also Chapter 7), a linear one compartment model sufficed for mathematical description of these data. Nevertheless, the non-linear approach outlined in Chapter 7, appeared to be of great help for meaningful interpretation of details of our findings, as will be shown throughout this chapter.

The most direct way to obtain information on the bioavailability of a drug is by comparison of oral and intravenous doses. Although there appears to be no therapeutic need for administering chlorthalidone intravenously, use of this dosage form in a fundamental pharmacokinetic study offers many advantages. Important kinetic constants, e.g. total plasma clearance and volumes of distribution, can be calculated without the uncertainty inherent to usage of oral doses. Moreover, by measuring the contribution of renal clearance of chlorthalidone to total clearance, ex-
istence of possible other routes of elimination will become established too. This aspect deserves attention, because e.g. the metabolic fate of chlor­thalidone in man has been completely disregarded until now, while species differences have been found in laboratory animals. Whereas the rat exten­sively metabolized the drug for ca. 70% of dose (Beisenherz et al., 1966), the dog excreted 90% of an oral dose unchanged into urine within 24 hours (Pulver et al., 1959). Some reviewers of the literature have extrapolated this resistance against metabolic breakdown to the human situation (Davies and Wilson, 1975; Swinyard, 1975), although not any actual evidence has been published that would justify such an assumption.

Measurement of both plasma, red blood cell and urine concentrations was considered necessary for reliable estimation of the bioavailability of chlorthalidone. Urinary excretion of the unchanged drug had to be deter­mined because possible changes in the balance between renal and non-renal clearance would obviously influence the estimate of bioavailability, if this was based upon plasma concentration data alone. Such variations had to be taken into account, due to the long biological half-life of chlorthalidone (see e.g. Chapter 7), which necessitated study over extended periods of time (several weeks). Erythrocyte concentrations had to be determined because the drug does not linearly distribute between plasma and red blood cells, as mentioned above, so that some disproportionality of the ratio of the areas under the curve after the two dosage modes had to be anticipated in case of incomplete oral bioavailability.

Determination of faecal concentrations of chlorthalidone, finally, was considered advantageous for discrimination between the extent of oral bioavailability, i.e. the fraction of dose coming into the systemic circulation, and the fraction of dose absorbed. A possible difference between these values should then be attributable to a first-pass effect by the liver. On the other hand, the amount of drug recovered in faeces after in­travenous administration would present information complementary to that to be described in Chapter 11 (Fleuren et al., 1979) on the biliary route of excretion of chlorthalidone in man.

The investigations described in this chapter were performed in a cross­over design, in which intravenous and oral doses of chlorthalidone were administered to seven normal human subjects.

MATERIALS AND METHODS

Subjects and drug administration

Seven adult human subjects (two females, five males) participated in the study after informed consent; personal data are given in Table 9.1. Three of them were healthy men (L.De, T.Ho, M.O.), who were - besides night’s
TABLE 9.1
Details of the subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Body weight (kg)</th>
<th>Plasma creatinine (μmol/l)</th>
<th>Diagnosis</th>
<th>Co-medication and daily doses during trial* (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Be</td>
<td>M</td>
<td>32</td>
<td>69</td>
<td>73</td>
<td>ulcer</td>
<td>aluminium hydroxide susp (Aluminox) 4×30 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cimetidine 5×200 mg, diazepam 3×5 mg</td>
</tr>
<tr>
<td>J v Bo</td>
<td>M</td>
<td>29</td>
<td>110</td>
<td>90</td>
<td>ulcer</td>
<td></td>
</tr>
<tr>
<td>L De</td>
<td>M</td>
<td>24</td>
<td>73</td>
<td>101</td>
<td>healthy</td>
<td></td>
</tr>
<tr>
<td>T Ho</td>
<td>M</td>
<td>24</td>
<td>76</td>
<td>90</td>
<td>healthy</td>
<td></td>
</tr>
<tr>
<td>A Kl</td>
<td>F</td>
<td>60</td>
<td>57</td>
<td>77</td>
<td>mental distress, borderline primary hypothyroidism cholelithiasis</td>
<td>flurazepam 1×30 mg</td>
</tr>
<tr>
<td>M O</td>
<td>M</td>
<td>28</td>
<td>83</td>
<td>87</td>
<td>healthy</td>
<td>acenocoumarol 4×1 mg, amoxycillin 3×750 mg, diazepam 3×5 mg, nitrazepam 5 mg, flucloxacillin 3×1000 mg</td>
</tr>
<tr>
<td>M We</td>
<td>F</td>
<td>43</td>
<td>62</td>
<td>77</td>
<td>spondylitis</td>
<td></td>
</tr>
</tbody>
</table>

* not on first half day of studies
rest - mainly ambulant or sitting during the trial, and carrying out their normal work (as students in the authors' laboratory). The four others were hospitalized for various reasons (see Table 9.1), but had normal clinical-chemical values* for their serum urea, creatinine, bilirubin, alkaline phosphatase, G.O.T., G.P.T., hematocrit, hemoglobin, plasma proteins and electro-cardiogram patterns. Therefore, all subjects were normal as far as their kidney, liver and heart functions were concerned.

Aseptic chlorthalidone solutions for intravenous administration were prepared in a concentration of 100 mg/l by dissolving pure chlorthalidone (kindly provided by Ciba-Geigy, Basle, Switzerland) in 0.9% pyrogen-free NaCl, which had been brought to pH 8.0 with a few drops of 0.1 M NaOH. After sterile filtration (Millipore, pore diameter 0.21 μm) a known volume was transferred to a graduated infusion flask. The drug solutions were prepared the day before the intravenous experiment and kept in the refrigerator until use, to prevent bacterial growth; chemically chlorthalidone was found to be stable in these solutions for at least four weeks, assayed by gas chromatography, as described in Chapter 3.

Each subject received a constant-rate infusion via a fore-arm antecubital vein over a period of approximately 2 hours (exact times in Table 9.3). An electronic drop counter (Ivac 531, I vac Corporation, San Diego, Ca., USA) was used to maintain zero-order infusion. The exact volume infused was calculated from the difference between the initial and remaining volumes in the flask. Five subjects received a dose of approximately 50 mg, one subject 60 mg and another 75 mg (exact amounts in Table 9.3), such that the doses applied were in the range of 0.66-0.88 mg per kg body weight. The infusions started in the morning at 9.00 A.M., after overnight fasting. The subjects took nothing except water during the first four hours of the study.

In the oral study commercial tablets were used (Hygroton®, Ciba-Geigy). For our purpose tablets containing 100 mg were divided and weighed proportionally until the desired amount was obtained, so that the doses were equal for each subject to those employed in the intravenous study (exact oral doses in Table 9.4). The tablets were ingested, crushed with the teeth and swallowed completely with about 150 ml of tap water, on an empty stomach, in the morning at 9.00 A.M. No food or beverages except water were taken until 4-5 hours after administration of the drug. During the first two hours, a volume of 0.9% NaCl (w/v) equal to the volume of fluid given in the intravenous study (500-750 ml, depending on the dose) was infused in order to exclude systematic differences in the fluid

* Determined at the Department of Clinical Chemistry, Radboud Hospital, Nijmegen, The Netherlands.
status of the subjects between the oral and the intravenous experiment. The
order of the administrations was such that four subjects received the oral
dose first (day nr. 1), followed by the intravenous dose (T.Be on day nr. 10,
J.v.Bo on day nr. 11, A.Kl on day nr. 8, M.We on day nr. 10). Three sub-
jects received the intravenous dose first (day nr. 1), followed by the tablet
(L.De on day nr. 15, M.O. on day nr. 36).

**Sampling and analytical measurements**

Venous blood samples (ca. 7 ml) were taken from the fore-arm, con-
tralateral to that of the infusion site, at the following approximate times
(exact times were noted) from the start of the infusion: 0, 0.5, 1, 1.5, 1.75,
2, 2.25, 2.5, 3, 4 and 5 hours. In the case of oral administration, samples
were taken at 0, 0.5, 0.75, 1, 1.5, 2, 3 and 4 hours. For both modes of ad-
ministration sampling was continued usually at 6, 8, 12, 24, 32, 48, 56, 72,
80, 96, 104 h after the start of the experiment, and from the subjects L.De,
T.Ho and M.O. additional blood samples at 171, 195 and 219 h were ob-
tained. Because of frequent sampling in the first 4 hours after the dose, the
antecubital vein was kept open by a slow-rate infusion (0.038 ml/min) of
physiological saline, containing heparin (0.5 ml Thromboliquine,
Organon, Oss, The Netherlands on 100 ml 0.9% NaCl), maintained by a
Harvard infusion pump (Harvard, Millis, Mass., USA). At the times
desired the tube was disconnected and the blood was collected directly
from the cannula, after discarding the first 1 ml. The remainder of the
samples was taken by vena punctum. All blood samples were heparinized
and centrifuged immediately after taking, (the detailed procedure stressing
the importance of an immediate separation of plasma from red blood cells
has been described in Chapter 3, Fleuren and van Rossum, 1978), and both
fractions were frozen until assay.

Urine was collected completely during 120 hours in separate, usually 6
hours portions from the hospitalized subjects and in separate 12 hours por-
tions during at least 220 hours (exact times in Table 9.7) by the three am-
bulant subjects. Urine volume and pH were measured and aliquots of each
portion were frozen until assay. The reliability of the completeness of the
urine collections was stated from urinary creatinine excretion, and addi-
tionally by a careful supervision and personal motivation of each subject
under trial.

Faeces were collected in 24 hours portions during 120 hours by the
hospitalized subjects, and during 220 hours by the ambulant subjects. The
portions were weighed, known volumes of distilled water were added and
the mixtures were thoroughly homogenized by the use of a Waring Blen-
dor. Aliquots were frozen until assay, or determined directly as follows: an
aliquot (about 0.1 gram) was weighed into an extraction tube, 2 ml of
Sörensen phosphate buffer pH 7.4 containing internal standard (5 μg, the

171
same compound as in Chapter 3) was added, the mixture was sonicated for 10 min and the supernatant was transferred, after brief centrifugation, to another tube. The sample was extracted and processed further identically as described for the assay of chlorthalidone in plasma (see below). As a control, calibration graphs with blank faeces from each subject were prepared, which proved that the extraction recoveries were the same as those obtained with buffer solutions. By performing the measurements in duplicate a relative standard deviation of 4% for the determination in faeces was obtained ($n = 8$).

The chlorthalidone concentrations in plasma, urine and red blood cells were determined by gas chromatography with nitrogen detection, as described in Chapter 3 (Fleuren and van Rossum, 1978). In one subject (M.We) the urine gas chromatograms showed two large interfering peaks, which we could prove to originate from the antibiotic flucloxacillin, used by this subject. Owing to the high water solubility of this compound, the interfering peaks could be removed by washing the methyl isobutyl ketone extracts twice with 2 ml of phosphate buffer pH 7.4, resulting in an excellent purification of these urine samples.

**Pharmacokinetics**

Preliminary graphical analyses of the plasma concentration versus time curves after intravenous and oral administration showed that the data could be described by either a two or a three compartment open model. Therefore the plasma concentrations were fitted to the corresponding sum of exponentials (Chapter 1):

**i.v. administration**

\[
C = \sum_{i=1}^{n} A_i \cdot \frac{1}{k_i \cdot T} (1 - e^{-k_i \cdot t}) \text{ for } t \leq T
\]  
(Eq.9.1)

and

\[
C = \sum_{i=1}^{n} A_i \cdot \frac{1}{k_i \cdot T} (e^{-k_i (t-T)} - e^{-k_i \cdot t}) \text{ for } t \geq T
\]  
(Eq.9.2)

**oral administration**

\[
C = \sum_{i=1}^{n} A_i \cdot (e^{-k_i \cdot t} - e^{-K \cdot t})
\]  
(Eq.9.3)
In these general equations C represents the plasma concentration, t is the
time after the start of the infusion or after the lagtime of the oral dose, T is
the infusion time, the coefficients $A_i$ are the hypothetical intercepts with
the ordinate, as in outlined in more detail in Chapter 1, $k_i$ refers to the $i$th
disposition rate constant (reciprocal time constant) where $i = 1\ldots n$, $K_a$
is the rate constant of first-order absorption into the central compartment
and $n$ is the number of compartments of the model employed.

After graphical analysis had yielded initial estimates, final parameter
values were obtained by use of the FARMFIT computer program*. A
relative error of 5% was attributed to each datapoint ($weight_i = 1/(0.05
C_i)^2$), as this was the standard deviation of the assay over the entire con­
centration range. The FARMFIT program provides a measure of the error
in a parameter estimate by dividing the asymptotic deviation of the
estimated parameter by the computer-estimated parameter itself (times 100
gives relative error in %). The choice of the value for the lagtime after oral
doses was not included in the regression analysis of the computer program
for two and three compartment models. Instead a preset value was used,
obtained after several prior estimations with different lagtime values,
yielding the lowest sum of weighed squared deviations, with negligibly
small improvement upon further change of this parameter.

Two criteria were used for each individual curve to decide between a two
or a three compartment model: 1) A visual inspection of the goodness of
fit, and 2) A statistical comparison of the fits by the f-ratio (Boxenbaum et
al., 1974). This test indicates if the total weighed sum of squared deviations
(SWSD) has been reduced sufficiently (for a certain level of significance,
e.g. 2.5%) in going from a two to a three compartment model, by taking-
into account the degrees of freedom (df) which remain after the choice of
the number of parameters defining the particular model (df = the number
of datapoints minus the number of parameters).

The biological half-life ($t^{1/2}$) was calculated from the smallest rate con­
stant ($k_i = \beta$) by $t^{1/2} = 0.693/\beta$.

The volume of the central compartment ($V_1$), the volume of distribution
at steady state ($V_{ds,ss}$) and the plasma clearance ($k_{cel}$) were calculated for
each curve directly from the coefficients and exponentials of the
appropriate exponential equation by use of the following relationships (van
Rossum, 1971; Wagner, 1976):

\[
v_1 = \frac{D}{n \sum_{i=1}^{n} A_i} \quad \text{ (Eq. 9.4)}
\]

* FARMFIT, a non-linear curve-fitting program, in use at the Computer Centre of the
University of Nijmegen. Details in Chapter 1.
oral administration

\[ V_1 = \frac{F.D}{n} \frac{1}{\Sigma (1-k_i/K_a) A_i} \]  
\[ (Eq.9.7) \]

\[ V_{d,ss} = \frac{F.D}{n} \frac{1}{(\Sigma (1-k_i/K_a) A_i/k_i)^2} \]  
\[ (Eq.9.8) \]

\[ k_{cel} = \frac{F.D}{n} \frac{1}{\Sigma (1-k_i/K_a) A_i/k_i} \]  
\[ (Eq.9.9) \]

where \( D \) is the dose, \( F \) is the available fraction, equal to the fraction of dose absorbed if no first-pass effect occurs, and the other symbols have the same meaning as explained for the Eq. 9.1, 9.2 and 9.3. As is evident from the Eq. 9.7, 9.8 and 9.9, only values for \( V_1/F \), \( V_{d,ss}/F \) and \( k_{cel}/F \) can be obtained if \( F \) is not actually known.

Because graphical plots of the erythrocyte concentration vs. time curves revealed a relatively slow and monophasic uptake of the drug into the
erythrocytes, both after the intravenous and the oral doses, followed by an apparently mono-exponential decay phase, the red blood cell concentrations after both modes of administration were fitted to a one compartment model with first-order absorption; for this Eq. 9.3 was used with $n = 1$ and $C$ representing here the erythrocyte concentration of chlorthalidone, and Eq. 9.7 yielded the volume of distribution. In case of a one compartment model the choice of a lagtime was made by the non-linear least squares regression analysis of the FARMFIT program. The maximum concentration reached in the red blood cells ($C_{\text{max}}$) and the time at which this occurred ($t_{\text{max}}$) was calculated routinely from the model parameters (Gibaldi and Perrier, 1975 a).

In addition to this separate approach to plasma and erythrocyte data, computer simulations were performed according to a non-linear model, which included the concentrations in plasma and erythrocytes simultaneously (Fleuren and van Rossum, 1977; Chapter 7). In this way the theoretical influence of a variation of dose upon the elimination half-life from plasma and erythrocytes and upon the ratio of the areas under the curve of a lower and a higher dose were studied, in order to see if non-linearities in this ratio would be apparent in the dose range encountered.

Estimates of bioavailability, i.e. the fraction of dose coming into the systemic circulation, were obtained from all three sets of data, viz. plasma, urine and erythrocyte measurements. The apparent bioavailability derived from the plasma and erythrocyte concentrations was calculated as follows (Gibaldi and Perrier, 1975 b):

$$\text{apparent bioavailability} = \frac{\text{AUC}_{\text{p.o.}} \cdot \text{D}_{\text{i.v.}} \cdot t_{\frac{1}{2}}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}} \cdot \text{D}_{\text{p.o.}} \cdot t_{\frac{1}{2}}_{\text{p.o.}}}$$  \quad (Eq. 9.10)

where the area under the curve (AUC) was obtained by use of the trapezoidal rule and extrapolation to infinity from the last datapoint, using the half-lives ($t_{\frac{1}{2}}$) resulting from the curve-fitting procedure.

The renal excretion rate plots were extrapolated to infinity from the actual analytical data by use of the half-life value, which had been observed from the decay of the red blood cell concentrations after oral administration for each individual subject, for reasons described in the Results section below. The ratios of the total amount of drug excreted in the urine over infinite time between the two modes of administration were calculated.

The renal clearance was calculated from each experiment by dividing the amount excreted in the urine during the period, that plasma concentrations had been measured, by the area under the plasma concentration curve obtained by the trapezoidal rule.
RESULTS

Plasma concentrations following intravenous administration

During the intravenous administration of chlorthalidone a regular rise in the plasma concentration occurred until the zero-order infusion was terminated. At that time peak concentrations between 0.44 and 0.87 µg/ml were measured. Immediately thereafter a rapid fall was observed with an apparent half-life between ca. 12 and 25 minutes, passing into a slower phase of disappearance with a mean half-life of 3.6 hours, until finally the elimination phase was reached with a long t½ between ca. 25 and 50 hours (mean 36.5 h).

In Figure 9.1 typical plasma concentration profiles are shown. All data-points of two subjects during and after infusion were fitted to the exponential equations for a two and a three compartment model. Visual inspection of the plots revealed the superiority of curve-fitting according to a three compartment model in five out of seven subjects; in one subject the extension of the number of compartments to three seemed doubtful (J.v.Bo) and in another (T.Be) no visual improvement at all was reached. The statistical criterion gave results in good agreement with the judgement-by-eye, as can be seen from Table 9.2: the f-ratio exceeded the critical value in the same five subjects, indicating a significant improvement in fit with the use of the three compartment model for them, whereas the test was negative with respect to the data of the subjects T.Be and J.v.Bo.

On the basis of these criteria it was concluded that a three compartment model was necessary for describing the time course of the plasma concentrations after intravenous administration in five subjects, while a two compartment model sufficed in the case of the two other subjects. The model parameters for the seven humans are presented in Table 9.3, together with the calculated values for the volume of the central compartment (V1), the volume of distribution at steady state (Vd,ss) and the plasma clearance (kce).

Plasma concentrations following oral administration

The time course of the plasma concentrations after chlorthalidone tablets was in agreement on the whole with previous observations (Fleuren and van Rossum, 1975; ibid., 1977; Chapter 7). In the present study, peak plasma concentrations of 0.14-0.26 µg/ml were measured approximately 1-3 hours after the dose. The decline of the curve thereafter consisted of two phases in all subjects, the first segment had an apparent half-life of ca. 2 h, the second part of the curve showed a decay with a mean t½ of 44 h. Similarly to the judgement-by-eye indicated the f-test that no improvement at all was obtained in the computer fits for any of the subjects when three compartments instead of two were used. Therefore, all data were calculated according to a two compartment open model, assuming first-
TABLE 9.5 A
Pharmacokinetic parameters describing chlortalidone concentration in erythrocytes after intravenous administration. The data were treated according to a one-compartment open model with first-order absorption. (Rel. errors in parentheses)

<table>
<thead>
<tr>
<th>Subject</th>
<th>T. Be</th>
<th>J. v. Bo</th>
<th>L. De</th>
<th>T. Ho</th>
<th>A. Kl</th>
<th>M. O</th>
<th>M. We</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{tv}$ (mg)</td>
<td>51.3</td>
<td>75.0</td>
<td>50.0</td>
<td>51.5</td>
<td>50.4</td>
<td>61.3</td>
<td>50.7</td>
</tr>
<tr>
<td>$t_{lag}$ (h)</td>
<td>0.32</td>
<td>0.37</td>
<td>0.39</td>
<td>0.31</td>
<td>0.24</td>
<td>0.33</td>
<td>0.34</td>
</tr>
<tr>
<td>(6.8%)</td>
<td>(4.8%)</td>
<td>(6.7%)</td>
<td>(5.8%)</td>
<td>(15.9%)</td>
<td>(5.6%)</td>
<td>(5.4%)</td>
<td></td>
</tr>
<tr>
<td>$K_a$ (h$^{-1}$)</td>
<td>0.534</td>
<td>0.454</td>
<td>0.470</td>
<td>0.56</td>
<td>0.445</td>
<td>0.387</td>
<td>0.579</td>
</tr>
<tr>
<td>(8.9%)</td>
<td>(9.6%)</td>
<td>(10.3%)</td>
<td>(6.2%)</td>
<td>(12.0%)</td>
<td>(7.4%)</td>
<td>(8.5%)</td>
<td></td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.0176</td>
<td>0.0149</td>
<td>0.0113</td>
<td>0.0146</td>
<td>0.0165</td>
<td>0.0124</td>
<td>0.0215</td>
</tr>
<tr>
<td>(4.9%)</td>
<td>(5.9%)</td>
<td>(4.1%)</td>
<td>(1.7%)</td>
<td>(8.1%)</td>
<td>(3.5%)</td>
<td>(3.3%)</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>39.3</td>
<td>46.7</td>
<td>61.4</td>
<td>47.5</td>
<td>42.1</td>
<td>55.7</td>
<td>32.2</td>
</tr>
<tr>
<td>$B$ (mg/l)</td>
<td>9.25</td>
<td>12.66</td>
<td>8.61</td>
<td>7.44</td>
<td>16.02</td>
<td>8.75</td>
<td>15.25</td>
</tr>
<tr>
<td>(4.6%)</td>
<td>(5.2%)</td>
<td>(4.8%)</td>
<td>(2.5%)</td>
<td>(6.2%)</td>
<td>(3.9%)</td>
<td>(4.0%)</td>
<td></td>
</tr>
<tr>
<td>$V_d$ (l)</td>
<td>5.73</td>
<td>6.13</td>
<td>5.95</td>
<td>7.11</td>
<td>3.27</td>
<td>7.24</td>
<td>3.45</td>
</tr>
<tr>
<td>(4.3%)</td>
<td>(4.7%)</td>
<td>(4.6%)</td>
<td>(2.4%)</td>
<td>(5.6%)</td>
<td>(3.6%)</td>
<td>(3.7%)</td>
<td></td>
</tr>
<tr>
<td>$C_{max}$ (mg/l)</td>
<td>7.97</td>
<td>10.90</td>
<td>7.67</td>
<td>6.58</td>
<td>13.6</td>
<td>7.56</td>
<td>12.9</td>
</tr>
<tr>
<td>$t_{max}$ (h)</td>
<td>6.61</td>
<td>7.78</td>
<td>8.13</td>
<td>5.92</td>
<td>7.69</td>
<td>9.19</td>
<td>5.91</td>
</tr>
</tbody>
</table>
TABLE 9.2

Fit of chlorthalidone plasma concentration versus time data after intravenous administration to two and three-component exponential equations. Statistical analysis of the weighted sums of squared deviations by the f-test

<table>
<thead>
<tr>
<th>Subject</th>
<th>Two-compartment model (four parameters)</th>
<th>Three-compartment model (six parameters)</th>
<th>f ratio*</th>
<th>Critical value of f (P=0.025)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df_i</td>
<td>SWSD_i</td>
<td>df_j</td>
<td>SWSD_j</td>
</tr>
<tr>
<td>T. Be</td>
<td>15</td>
<td>138.3</td>
<td>13</td>
<td>117.6</td>
</tr>
<tr>
<td>J. v. Bo</td>
<td>17</td>
<td>38.0</td>
<td>15</td>
<td>23.7</td>
</tr>
<tr>
<td>L. De</td>
<td>19</td>
<td>106.7</td>
<td>17</td>
<td>37.9</td>
</tr>
<tr>
<td>T. Ho</td>
<td>18</td>
<td>133.1</td>
<td>16</td>
<td>36.2</td>
</tr>
<tr>
<td>A. Kl</td>
<td>15</td>
<td>157.5</td>
<td>13</td>
<td>82.5</td>
</tr>
<tr>
<td>M. O</td>
<td>18</td>
<td>80.7</td>
<td>16</td>
<td>23.5</td>
</tr>
<tr>
<td>M. We</td>
<td>16</td>
<td>121.3</td>
<td>14</td>
<td>54.8</td>
</tr>
</tbody>
</table>

df = remaining degrees of freedom
SWSD = sum of weighted squared deviations

\[ f* = \left( \frac{\text{SWSD}_i - \text{SWSD}_j}{\text{SWSD}_j} \right) \times \left( \frac{\text{df}_i}{\text{df}_i - \text{df}_j} \right) \]
<table>
<thead>
<tr>
<th></th>
<th>Three compartments</th>
<th>Two compartments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subject</td>
<td></td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>L De 50.0</td>
<td>T Be 51.3</td>
</tr>
<tr>
<td></td>
<td>T Ho 51.5</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>A Kl 50.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M O 61.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M We 50.7</td>
<td></td>
</tr>
<tr>
<td>Dose/kg (mg/kg)</td>
<td>0.69</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>0.68</td>
<td>0.82</td>
</tr>
<tr>
<td>Infusion time (h)</td>
<td>2.00</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2.17</td>
</tr>
<tr>
<td>α (h⁻¹)</td>
<td>2.18 (19.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.12 (15.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(29.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(18.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(42.9%)</td>
<td></td>
</tr>
<tr>
<td>β (h⁻¹)</td>
<td>0.217 (37.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.133 (33.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(58.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(30.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(44.0%)</td>
<td></td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>49.8 (11.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44.2 (11.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.6 (19.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46.7 (8.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.8 (29.1%)</td>
<td></td>
</tr>
<tr>
<td>P (mg/l)</td>
<td>1.09 (11.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.28 (11.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.40 (19.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.02 (8.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.15 (29.1%)</td>
<td></td>
</tr>
<tr>
<td>A (mg/l)</td>
<td>0.116 (36.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.119 (21.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.221 (26.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.154 (33.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.224 (41.0%)</td>
<td></td>
</tr>
<tr>
<td>B (mg/l)</td>
<td>0.106 (4.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.119 (6.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.142 (31.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.121 (3.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.179 (9.8%)</td>
<td></td>
</tr>
<tr>
<td>V (l)</td>
<td>38.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.9 (6.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.6 (31.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>47.4 (3.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.8 (9.8%)</td>
<td></td>
</tr>
<tr>
<td>Vₐₛ (l)</td>
<td>366</td>
<td></td>
</tr>
<tr>
<td></td>
<td>306</td>
<td></td>
</tr>
<tr>
<td></td>
<td>198</td>
<td></td>
</tr>
<tr>
<td></td>
<td>388</td>
<td></td>
</tr>
<tr>
<td></td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>kₐₛ (l/h)</td>
<td>5.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.63</td>
<td></td>
</tr>
</tbody>
</table>
order absorption into and first-order elimination from the central compartment.

The resulting pharmacokinetic parameters are shown in Table 9.4. By comparison with Table 9.3 it can be seen that the terminal $t_{1/2}$ of three subjects (T.Be, J.v.Bo, A.Kl) is substantially longer than that observed with them in the intravenous study. Furthermore, it should be noticed that the values for the volume of the central compartment, the total volume of distribution at steady state and the plasma clearance can be obtained only in a relative sense from oral administration data, viz. divided by the bioavailability ($F$).

**Red blood cell concentrations after intravenous and oral administration**

The rise of the erythrocyte concentrations of chlorthalidone was relatively slow compared to that of the initial plasma concentrations, even after the intravenous dose, where maximum concentrations were not reached until ca. 6-9 hours after the start of the infusion.

Upon oral administration the highest values were found between approximately 8 and 16 hours after the dose, similarly to earlier results (Fleuren and van Rossum, 1975; ibid., 1977; Chapter 7). The times at which the maxima occurred coincided reasonably with the end of the distribution phases of the plasma concentrations.

After the top of the curves, the decline of the red blood cell level was mono-exponential for the oral dosage form in all seven subjects. Therefore, the oral data were fitted to a one compartment model with first-order absorption and the resulting pharmacokinetic parameters are shown in Table 9.5 B.

After the intravenous dose of chlorthalidone, a substantially faster decay than after the oral dose was observed in four subjects, three of whom had also shown a faster disappearance of their plasma concentrations in the intravenous study (T.Be, J.v.Bo, A.Kl). During the ca. 100 hour-period, in which the blood samples had been obtained from them, no appreciable deviation from a straight line was apparent when the concentrations were plotted on a semi-logarithmic scale. A typical illustration of this phenomenon is given in Figure 9.2, where the upper part represents the concentration versus time curves in the erythrocytes and the lower part shows the concentrations in plasma, followed in the same subject after both dosage modes. It can be seen that the $t_{1/2}$ of the oral dose did not change after the first 100 hour-period, judged from the concentration at 219 h (analyzed in duplicate), which arose from the assay of the zero-point before the next dose ($t = 0$ h of the intravenous dose)*. Similarly, the late oral datapoints from the subjects A.Kl, M.We and T.Be were located within a few percent of the fitted curves, based upon the first 100 hours only.
TABLE 9.4
Pharmacokinetic parameters describing plasma concentration of chlorthalidone after oral administration to seven human subjects according to a two-compartment open model. (Rel. errors in parentheses)

<table>
<thead>
<tr>
<th>Subject</th>
<th>T. Be</th>
<th>J. v. Bo</th>
<th>L. De</th>
<th>T. Ho</th>
<th>A. Kl</th>
<th>M. O</th>
<th>M. We</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg)</td>
<td>50.0</td>
<td>75.0</td>
<td>50.0</td>
<td>50.4</td>
<td>50.0</td>
<td>60.9</td>
<td>50.0</td>
</tr>
<tr>
<td>Dose/weight (mg/kg)</td>
<td>0.73</td>
<td>0.68</td>
<td>0.69</td>
<td>0.66</td>
<td>0.87</td>
<td>0.73</td>
<td>0.81</td>
</tr>
<tr>
<td>Lag time (h)</td>
<td>0.25</td>
<td>0.35</td>
<td>0.40</td>
<td>0.60</td>
<td>0.70</td>
<td>0.40</td>
<td>0.75</td>
</tr>
<tr>
<td>$K_a$ (h$^{-1}$)</td>
<td>5.79</td>
<td>1.22</td>
<td>1.53</td>
<td>1.51</td>
<td>2.73</td>
<td>1.54</td>
<td>1.29</td>
</tr>
<tr>
<td>(22.3%)</td>
<td>(45.5%)</td>
<td>(21.6%)</td>
<td>(11.9%)</td>
<td>(10.1%)</td>
<td>(20.9%)</td>
<td>(39.0%)</td>
<td></td>
</tr>
<tr>
<td>$\alpha$ (h$^{-1}$)</td>
<td>0.205</td>
<td>0.75</td>
<td>0.131</td>
<td>0.343</td>
<td>0.242</td>
<td>0.190</td>
<td>0.87</td>
</tr>
<tr>
<td>(12.5%)</td>
<td>(47.1%)</td>
<td>(46.5%)</td>
<td>(12.9%)</td>
<td>(16.3%)</td>
<td>(30.0%)</td>
<td>(26.0%)</td>
<td></td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.0157</td>
<td>0.0144</td>
<td>0.0136</td>
<td>0.0161</td>
<td>0.0137</td>
<td>0.0145</td>
<td>0.0293</td>
</tr>
<tr>
<td>(7.8%)</td>
<td>(5.8%)</td>
<td>(5.4%)</td>
<td>(2.2%)</td>
<td>(3.7%)</td>
<td>(2.9%)</td>
<td>(2.5%)</td>
<td></td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>44.2</td>
<td>48.2</td>
<td>51.1</td>
<td>42.9</td>
<td>50.5</td>
<td>47.9</td>
<td>23.7</td>
</tr>
<tr>
<td>A (mg/l)</td>
<td>0.177</td>
<td>1.04</td>
<td>0.099</td>
<td>0.393</td>
<td>0.198</td>
<td>0.104</td>
<td>1.10</td>
</tr>
<tr>
<td>(8.1%)</td>
<td>(96.0%)</td>
<td>(31.6%)</td>
<td>(17.5%)</td>
<td>(11.7%)</td>
<td>(28.4%)</td>
<td>(42.8%)</td>
<td></td>
</tr>
<tr>
<td>B (mg/l)</td>
<td>0.0555</td>
<td>0.0940</td>
<td>0.0895</td>
<td>0.0975</td>
<td>0.103</td>
<td>0.0764</td>
<td>0.0981</td>
</tr>
<tr>
<td>(8.6%)</td>
<td>(5.4%)</td>
<td>(9.5%)</td>
<td>(3.6%)</td>
<td>(4.5%)</td>
<td>(5.1%)</td>
<td>(3.5%)</td>
<td></td>
</tr>
<tr>
<td>$V_1/F$ (l)</td>
<td>221</td>
<td>152</td>
<td>280</td>
<td>126</td>
<td>176</td>
<td>366</td>
<td>110</td>
</tr>
<tr>
<td>$V_{ss}/F$ (l)</td>
<td>602</td>
<td>690</td>
<td>467</td>
<td>399</td>
<td>402</td>
<td>681</td>
<td>424</td>
</tr>
<tr>
<td>$k_{ce}/F$ (l/h)</td>
<td>11.5</td>
<td>10.7</td>
<td>6.92</td>
<td>7.35</td>
<td>6.06</td>
<td>10.7</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Footnote of page 180:
* The concentrations in the second experiment were corrected for the remainder of the previous dose by subtracting an amount calculated from the zero-time concentration and the half-life actually observed during the next dose. This correction was no greater than 4.0% of the total area under the curve for subject J.v.Bo (1.3% T.Be, 6.7% A.Kl, 0.4% M.We).
Comparison of the time course of plasma and erythrocyte concentrations of chlorthalidone after an equal intravenous and oral dose to the same human subject. The measurements in both the red blood cells (upper graph) and in the plasma (lower graph) show a faster disappearance of the concentrations after the intravenous dose. The pictures have been taken directly from computer plots, obtained by fitting the data to their appropriate pharmacokinetic equations.

In the intravenous study in another subject (T.Ho), from whom blood samples had been taken up to ca. 220 hours, a slightly faster decay was observed in roughly the first half of the curve, which passed over gradually to the same decline as that estimated after the oral dose. In two other subjects where also a long sampling period was employed (L.De and M.O.) the time course of the red blood cell concentrations after the intravenous dose was apparently mono-exponential, remaining approximately parallel to the curve of the oral study. Examples of both kinds of decay are shown in Figure 9.3.

Figure 9.2
Comparison of the time course of plasma and erythrocyte concentrations of chlorthalidone after an equal intravenous and oral dose to the same human subject. The measurements in both the red blood cells (upper graph) and in the plasma (lower graph) show a faster disappearance of the concentrations after the intravenous dose. The pictures have been taken directly from computer plots, obtained by fitting the data to their appropriate pharmacokinetic equations.
Figure 9.3
Typical red blood cell concentration profiles of chlorthalidone on semi-logarithmic scale after approximately equal intravenous and oral doses in two human subjects. A slightly faster decay in the intravenous study, caused mainly by the first part of the period, was observed here in one subject (upper panel), whereas almost equal half-lives for both modes of administration were found in the other (lower panel).
Pharmacokinetics of chlorthalidone in plasma after a single intravenous dose in two normal human subjects, followed during ca. 200 hours. The upper and lower graphs show computer fits according to, respectively, a two and a three compartment model. The inset pictures, representing the plasma concentrations during the first 35 hours at an expanded scale, demonstrate the superiority of the three compartment fits.
Cumulative urinary excretion of unchanged chlorthalidone after equal intravenous and oral doses to the same human subjects. From 26.5-51.8% of dose was totally excreted after orally (●), and from 50.1-77.7% of dose after intravenously (○) administered chlorthalidone.

**Faecal excretion**

Measurable concentrations of chlorthalidone in human faeces (above ca. 20 ng/sample) were present during a period varying from 4-8 days after the intravenous or oral dose. The peaks in the excretion mostly occurred during the second day after intravenous administration and from 1-3 days after the oral dose, the actual excretion rate of each individual naturally being dependent upon the regularity of the defaecation pattern. The individual data are presented in Table 9.8. During the intravenous study total amounts of 1.3-8.5% of the dose were excreted, whereas after oral administration 17.5-31% of the dose was found in faeces.

**DISCUSSION AND CONCLUSIONS**

As far as we know, no reports concerning the intravenous administration of this drug to humans have appeared in the literature. The drug was dissolved in physiological saline because no untoward effects were expected from this dosage form, when administered at a sufficiently slow rate. It was possible, therefore, to take a 2 hours period for the infusion of the
In the calculation of bioavailability, half-life differences can be handled by using a correction for them, based upon a constant clearance of the drug during both periods of measurements (Gibaldi and Perrier, 1975 b). The most straightforward way was to consider the clearance to be constant over every period of measurement, even if it was anticipated that the half-lives after intravenous administration would be only temporarily smaller and would change to larger values after the actual time of analysis, as suggested by the case of subject T.Ho (see Figure 9.3). The erythrocyte concentrations in all subjects were therefore forced into a one compartment model, which seemed to be allowed because of a reasonable agreement between the actual data and the fitted curves, which is shown in the Figures 9.2 and 9.3, even with the intravenous dose in subject T.Ho, in whom the difference between the initial and final elimination rate was too small to permit the use of a more complicated model.

The resulting pharmacokinetic parameters are presented in Table 9.5. Comparison with the results after the oral dose, given in the same Table, shows that the estimated half-lives in five subjects (T.Be, J.v.Bo, T.Ho, A.Ki, M.We) were shorter in the intravenous study than in the oral study; the relatively small magnitude of the difference in two subjects (T.Be, T.Ho) was the overall result of a small but discernible change in elimination rate during the time of analysis, see e.g. Fig. 9.3. Table 9.5 reveals furthermore that the individual values for the apparent lagtime ($t_{lag}$) were smaller and for the absorption constant ($K_a$) were larger in the intravenous study, corresponding to the earlier times at which the maxima were reached.

**Ratio of the areas under the plasma and red blood cell concentration curves of oral versus intravenous doses of chlorthalidone**

Figure 9.4 shows individual plasma concentration data of chlorthalidone after intravenous and oral administration of this drug to six human subjects (the data of the seventh subject, A.Ki, have been shown in another context, in Fig. 2.4, Chapter 2). Comparison of the oral and intravenous dosage form yielded a mean apparent bioavailability of 0.61 (individual values in Table 9.6A), whereas a mean apparent ratio of 0.72 was found from the erythrocyte concentration curves (individual values in Table 9.6B).

**Computer simulations according to a non-linear chlorthalidone model**

Calculations performed on several sets of concentration-time curves showed that the bioavailability assessments based upon red blood cell concentrations would yield about 10% too high values for the oral dose. As an example, when simulations with the fitting model-parameters, given in Chapter 7 (Fig. 7.7), were carried out (with $F = 0.25$ arbitrarily, the dose thus being 50 mg), lowering the dose to three-fourth part did not result in a
Figure 9.4
Plots showing the areas under the plasma concentration vs time curves of chlorthalidone after equal intravenous (○) and oral (●) doses. From these data, a bioavailability ranging from 47-81% was calculated for the oral dosage form.

area under the curve ratio of 0.75, expected in case of linear kinetics, but instead the outcome was 0.83, when calculated according to Eq. 9.10, while the t½ (32-100 h) changed only from 70 to 73 h. This non-proportional increase in area under the curve was due to the capacity-limited, strong bin-
TABLE 9.6
Estimation of the bioavailability of chlorthalidone by comparison of the areas under the curves after intravenous and oral doses in seven normal human subjects. Upper part (A) plasma concentrations. Lower part (B) erythrocyte concentrations.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time period (t) measured</th>
<th>Half-life (h)</th>
<th>AUC plasma 0–t (h mg/l)</th>
<th>AUC plasma at infinity (h mg/l)</th>
<th>Bioavailability estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Be</td>
<td>p o 104</td>
<td>44.2</td>
<td>3.79</td>
<td>4.74</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>i v 96</td>
<td>25.5</td>
<td>5.44</td>
<td>5.79</td>
<td></td>
</tr>
<tr>
<td>J v Bo</td>
<td>p o 104</td>
<td>48.2</td>
<td>5.56</td>
<td>7.03</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>i v 96</td>
<td>30.8</td>
<td>8.42</td>
<td>9.53</td>
<td></td>
</tr>
<tr>
<td>L De</td>
<td>p o 219</td>
<td>51.1</td>
<td>6.89</td>
<td>7.26</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>i v 219</td>
<td>49.8</td>
<td>8.40</td>
<td>8.78</td>
<td></td>
</tr>
<tr>
<td>T Ho</td>
<td>p o 195</td>
<td>42.9</td>
<td>6.78</td>
<td>7.04</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>i v 195</td>
<td>44.2</td>
<td>8.88</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td>A Kl</td>
<td>p o 192</td>
<td>50.5</td>
<td>7.88</td>
<td>8.42</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>i v 80</td>
<td>34.6</td>
<td>8.43</td>
<td>9.89</td>
<td></td>
</tr>
<tr>
<td>M O</td>
<td>p o 219</td>
<td>47.9</td>
<td>5.58</td>
<td>5.81</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>i v 195</td>
<td>46.7</td>
<td>8.92</td>
<td>9.39</td>
<td></td>
</tr>
<tr>
<td>M We</td>
<td>p o 80</td>
<td>23.7</td>
<td>3.33</td>
<td>3.66</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>i v 104</td>
<td>23.8</td>
<td>7.60</td>
<td>7.92</td>
<td></td>
</tr>
</tbody>
</table>

**mean (±SD)**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time period (t) measured</th>
<th>Half-life (h)</th>
<th>AUC erythr 0–t (h mg/l)</th>
<th>AUC erythr at infinity (h mg/l)</th>
<th>Bioavailability estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Be</td>
<td>p o 104</td>
<td>44.6</td>
<td>364</td>
<td>457</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>i v 96</td>
<td>39.3</td>
<td>407</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td>J v Bo</td>
<td>p o 240</td>
<td>58.9</td>
<td>708</td>
<td>751</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>i v 104</td>
<td>46.7</td>
<td>635</td>
<td>817</td>
<td></td>
</tr>
<tr>
<td>L De</td>
<td>p o 219</td>
<td>60.0</td>
<td>597</td>
<td>653</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>i v 219</td>
<td>61.4</td>
<td>671</td>
<td>734</td>
<td></td>
</tr>
<tr>
<td>T Ho</td>
<td>p o 219</td>
<td>50.0</td>
<td>415</td>
<td>437</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>i v 219</td>
<td>47.5</td>
<td>474</td>
<td>496</td>
<td></td>
</tr>
<tr>
<td>A Kl</td>
<td>p o 192</td>
<td>64.1</td>
<td>699</td>
<td>810</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>i v 80</td>
<td>42.1</td>
<td>678</td>
<td>947</td>
<td></td>
</tr>
<tr>
<td>M O</td>
<td>p o 219</td>
<td>52.2</td>
<td>397</td>
<td>421</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>i v 195</td>
<td>55.7</td>
<td>618</td>
<td>686</td>
<td></td>
</tr>
<tr>
<td>M We</td>
<td>p o 264</td>
<td>38.8</td>
<td>404</td>
<td>407</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>i v 104</td>
<td>32.2</td>
<td>596</td>
<td>678</td>
<td></td>
</tr>
</tbody>
</table>

**mean (±SD)**

0.61±0.15
ding of chlorthalidone to erythrocytes at higher concentrations. In the converse case, the plasma concentration measurements yielded smaller than proportional areas after the lower dose, but the difference between the known bioavailability (0.75) and the calculated value (0.74) was much less than that found with the erythrocyte concentrations. This was attributed to the compensatory effect of first-order elimination, taking place from the central compartment to which the plasma volume belongs. The $t/2$ of the plasma concentrations changed only from 42 to 44 hours when the dose was reduced.

**Urinary excretion of chlorthalidone after intravenous and oral administration**

The urinary excretion rate versus time plots ran parallel to the decay of the plasma concentrations, as long as the latter could be accurately analyzed (down to ca. 5 ng/ml, using 2 ml of plasma). After that time there was a tendency for all graphs to accept gradually the $t/2$ of the erythrocyte concentrations observed after oral administration. Typical examples are given in the Figures 9.5 and 9.6, where the urinary excretion was followed in two human subjects, in one during 120 hours and additionally from 215-239 hours and in the other during 220 hours and from 292-335 hours after the dose. The excretion rate during the later time was in every case above the value expected from the regression line of the first period. The total amount of unchanged drug excreted at infinite time was therefore calculated by extrapolation with the use of this oral half-life value after the actual period of analysis. This was preferred firstly because very precise half-life values from renal excretion data can be obtained only if the urine samples have been collected very frequently and considerable fluctuations in renal clearance within and between days do not occur. These requirements were not met in the present study, because urine was collected during 6 or 12 hour-periods and some subjects showed a remarkable variation in excretion rate, often coinciding with changes in urine flow, which is shown in Fig. 9.5. Secondly, according to the non-linear chlorthalidone disposition model (Chapter 7), the plasma concentration decay and therefore, under the assumption of first-order renal elimination, also the urinary excretion rate should become parallel ultimately to the red blood cell concentration decay after a period of time long enough to reach appropriately low concentrations.

Table 9.7 summarizes the urinary excretion data of all subjects after both modes of administration of chlorthalidone. The actual cumulative amounts excreted during the sampling period are given as well as the total calculated amounts, obtained by extrapolation to infinite time. See also Figure 9.7. After intravenous administration of chlorthalidone a mean value of 65.4% of the dose was excreted as unchanged drug at infinite time.
and a mean value of 43.8% was found after the oral dose. The bioavailability of the oral dose was calculated as the ratio of these total amounts excreted, resulting in a mean value of 67%.

Renal clearance of chlorthalidone ranged from 43.5-97.7 ml/min (Table 9.7). Except for a rather large difference in one subject (A.KI), the values in the whole group of subjects were not systematically higher or lower after either of the two dosage modes.

![Graph](image)

**Figure 9.5 (left panel)**
Renal excretion rate of chlorthalidone versus time. To facilitate comparison with the plasma concentration curve of the same subject (t½ = 48.2 h) a regression line has been drawn based upon the average 24 h excretion rate during 24-120 hours, indicated as open circles. The half-life calculated from the last point of this period and the later data-point at 227 h (t½ ca. 57 h) approximates the longer t½ of the erythrocyte concentrations after oral administration. In the lower graphs the urine flow and the pH of the urine fractions are shown.

**Figure 9.6 (right panel)**
Urinary excretion rate of chlorthalidone versus time after a single 51.5 mg intravenous dose in man. The excretion rate is parallel with the observed decay of the plasma concentrations in the subject (t½ = 44.2), shown by regression of the averaged daily excretion rate values from 24-220 hours (open circles). The half-life calculated from the later period of assay, 292-335 hours, and the last point of the first period (t½ ca. 52 h) apparently equals the t½ of the red blood cell concentrations after oral administration (t½ = 50 h in this subject). The total amount of drug excreted unchanged at infinite time was 72 percent of the intravenous dose.
TABLE 9.7

Urinary excretion of chlorthalidone after intravenous and oral administration of equal (usually 50 mg) doses to the same seven human subjects. Calculation of renal clearance and estimation of oral bioavailability

<table>
<thead>
<tr>
<th>Subject and mode of administration</th>
<th>Time period measured (h)</th>
<th>Cumulative excretion of chlorthalidone</th>
<th>Bioavailability estimate</th>
<th>Renal clearance (l/h)</th>
<th>Urine flow (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>During assay amount (mg)</td>
<td>% of dose</td>
<td>At infinite time(^a) amount (mg)</td>
<td>% of dose</td>
</tr>
<tr>
<td>T. Be p. o.</td>
<td>119</td>
<td>22.96</td>
<td>45.9</td>
<td>25.91</td>
<td>51.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.15</td>
<td>58.8</td>
<td>32.85</td>
<td>64.0</td>
</tr>
<tr>
<td>J. v. Bo p. o.</td>
<td>119</td>
<td>26.83</td>
<td>35.8</td>
<td>32.34</td>
<td>43.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44.6</td>
<td>59.5</td>
<td>48.16</td>
<td>64.2</td>
</tr>
<tr>
<td>L. De p. o.</td>
<td>220</td>
<td>22.15</td>
<td>44.3</td>
<td>23.82</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.6</td>
<td>63.2</td>
<td>33.65</td>
<td>67.3</td>
</tr>
<tr>
<td>T. Ho p. o.</td>
<td>220</td>
<td>24.81</td>
<td>49.2</td>
<td>25.6</td>
<td>50.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.89</td>
<td>69.7</td>
<td>37.1</td>
<td>72.0</td>
</tr>
<tr>
<td>A. Kl p. o.</td>
<td>95</td>
<td>15.94</td>
<td>31.9</td>
<td>21.32</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.23</td>
<td>64.0</td>
<td>39.2</td>
<td>77.7</td>
</tr>
<tr>
<td>M. O. p. o.</td>
<td>296</td>
<td>26.39</td>
<td>43.3</td>
<td>26.92</td>
<td>44.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.03</td>
<td>60.4</td>
<td>38.43</td>
<td>62.7</td>
</tr>
<tr>
<td>M. We p. o.</td>
<td>119</td>
<td>12.3</td>
<td>24.6</td>
<td>13.25</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.62</td>
<td>48.6</td>
<td>25.40</td>
<td>50.1</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) extrapolation to infinity was performed by use of the half-life of the erythrocyte concentration after oral administration. For further explanation see text.

\(^b\) very similar renal clearance values were obtained when only the first 48-h-period was used.
Table 9.5B
Pharmacokinetic parameters describing chlorthalidone concentration in erythrocytes after oral administration. The data were treated according to a one-compartment open model with first-order absorption (Rel. errors in parentheses).

<table>
<thead>
<tr>
<th>Subject</th>
<th>D_{p0} (mg)</th>
<th>t_{lag} (h)</th>
<th>K_a (h⁻¹)</th>
<th>β (h⁻¹)</th>
<th>t_{½} (h)</th>
<th>B (mg/l)</th>
<th>V_{d/F} (l)</th>
<th>C_{max} (mg/l)</th>
<th>t_{max} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Be</td>
<td>50.0</td>
<td>0.34</td>
<td>0.385</td>
<td>0.0155</td>
<td>44.6</td>
<td>7.40</td>
<td>7.04</td>
<td>6.21</td>
<td>8.69</td>
</tr>
<tr>
<td>Jv Bo</td>
<td>75.0</td>
<td>0.51</td>
<td>0.313</td>
<td>0.0118</td>
<td>58.9</td>
<td>8.70</td>
<td>8.96</td>
<td>7.36</td>
<td>10.88</td>
</tr>
<tr>
<td>L De</td>
<td>50.0</td>
<td>0.46</td>
<td>0.180</td>
<td>0.0116</td>
<td>60.0</td>
<td>7.95</td>
<td>6.72</td>
<td>6.16</td>
<td>16.3</td>
</tr>
<tr>
<td>T Ho</td>
<td>50.4</td>
<td>0.64</td>
<td>0.316</td>
<td>0.0138</td>
<td>50.0</td>
<td>6.33</td>
<td>8.32</td>
<td>5.26</td>
<td>10.3</td>
</tr>
<tr>
<td>A Kl</td>
<td>50.0</td>
<td>0.82</td>
<td>0.29</td>
<td>0.0108</td>
<td>64.1</td>
<td>8.85</td>
<td>5.87</td>
<td>7.50</td>
<td>11.8</td>
</tr>
<tr>
<td>MO</td>
<td>60.9</td>
<td>0.40</td>
<td>0.195</td>
<td>0.0133</td>
<td>52.2</td>
<td>5.98</td>
<td>10.9</td>
<td>4.59</td>
<td>14.8</td>
</tr>
<tr>
<td>M We</td>
<td>50.0</td>
<td>0.89</td>
<td>0.437</td>
<td>0.0179</td>
<td>38.8</td>
<td>7.04</td>
<td>7.41</td>
<td>5.89</td>
<td>7.62</td>
</tr>
</tbody>
</table>
Table 9.8
Excretion of chlorthalidone in faeces after intravenous (i.v) and oral (p.o.) administration of equal (usually 50 mg) doses to the same human subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time period measured (h)</th>
<th>Cumulative amount (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p.o</td>
</tr>
<tr>
<td>T Be</td>
<td>120</td>
<td>17.5</td>
</tr>
<tr>
<td>J v Bo</td>
<td>120</td>
<td>21.0</td>
</tr>
<tr>
<td>L De</td>
<td>220</td>
<td>24.2</td>
</tr>
<tr>
<td>T Ho</td>
<td>220</td>
<td>20.9</td>
</tr>
<tr>
<td>M O</td>
<td>220</td>
<td>31.2</td>
</tr>
<tr>
<td>M We</td>
<td>120</td>
<td>n.d</td>
</tr>
</tbody>
</table>

n.d = not determined

Volume of fluid, necessary because of the limited solubility of chlorthalidone in neutral aqueous solutions (Stenger et al., 1959; Chapter 6).

Upon questioning, the subjects under study stated that no effects, except upon urine production, were experienced by them during and after the infusion.

Curve-fitting according to 3 versus 2 compartment models

With respect to the plasma concentrations of chlorthalidone after intravenous administration a perfect agreement was noticed between the results of the f-test and of the visual inspection of the goodness of fit. Both criteria indicated the superiority of the three compartment model in five out of seven subjects and a serious underestimation of the terminal half-life would have occurred by the use of the two compartment model. Table 9.3 shows that relatively great errors were found for the parameter estimates describing the first and second phase of distribution after the intravenous dose, due to a limited number of datapoints in these segments, despite reasonable visual fits, see e.g. Figure 9.1. In contrast, errors in the oral absorption rate constants obviously reflected also deviations in some subjects from a purely first-order absorption process, which, however, remained the most effective approach to include the data. Good accuracy was obtained for the estimates of the terminal elimination rate constants and, therefore, also of the terminal half-lives. Comparison of the Tables 9.3 and 9.4 showed that the half-lives after intravenous administration were shorter than those after the oral dose in five of the seven humans. Simulations with a non-linear model indicated that differences of such magnitude were not expected to follow from a less tight binding of the drug to red blood cells at the higher concentrations reached initially with the intravenous dose.
Moreover, the concentration range in which the half-life differences became evident was practically the same for both dosage forms, see e.g. Figure 9.2. It is reasonable therefore to understand the shorter half-lives of plasma and erythrocyte concentrations as being due to an enlarged clearance of chlorthalidone after intravenous administration, assuming that the volume of distribution remained the same during both trials. Inspection of Figure 9.5 might suggest that the faster elimination could have been caused by the larger urine flows, generally observed after the intravenous doses, see Table 9.7. Surprisingly, however, no proportional increase, except in one case, in the time-averaged renal clearance was evident in the subjects where shorter half-lives after the intravenous dose were found, see Table 9.7, implying that the above difference should become mainly on account of increases in non-renal clearance.

**Bioavailability measurements**

For a definite statement of a greater plasma clearance after intravenous doses than after oral medication the exact values of the bioavailability $F$ have to be known. This factor $F$ in turn can be determined reliably from urinary excretion data only, if the balance between renal and non-renal clearance had not changed between the oral and the intravenous trials. Thus, this kind of a vicious circle presents a serious limitation for the exact interpretation of the data in some subjects. Nevertheless, the disagreement for two subjects between the bioavailability calculations based upon urinary excretion data and plasma concentrations (see Tables 9.6 A and 9.7) could be explained by an increased non-renal clearance after intravenous administration, such that a greater proportion of drug was eliminated by another route than by excretion in the urine. Consequently the oral bioavailability calculated from the urinary excretion data in these subjects ($T.Be$, $J.v.Bo$) was too high.

On the other hand it seemed unwise to rely upon plasma data only, because the semi-logarithmic plots of the elimination phase were, though reasonably, not perfectly linear, see e.g. Figure 9.2, indicating a decreasing clearance already during the measuring period, so that the model used should be considered only approximative in these cases. In the absence of a more detailed explanation for the differences in $t_{\frac{1}{2}}$ than described above, we decided not to prefer either the urinary excretion data or the plasma concentration data for assessment of the bioavailability of chlorthalidone, and when weighing equally both sets of data a mean absolute bioavailability $F = 0.64$ results for the oral doses.

In contrast, the bioavailability estimate based upon red blood cell concentration data yields too high values, judged from computer simulations, and supported further by the observation that the sum of the apparently available dose and dose remaining in the gastro-intestinal tract after oral
administration (see Tables 9.6 B and 9.8) exceeded 100% in two subjects (L.De, T.Ho).

In principle, a decreased oral bioavailability can be caused by limited absorption, by a so-called "first-pass effect" in the liver or by a combination of both processes. Judged from the total amounts of drug recovered from the faeces after oral doses, limited absorption must be the predominant factor here, as only minor amounts were found in the faeces after intravenous administration, ruling out the possibility of considerable transport back into the gut lumen under normal conditions. However, comparison of the amounts non-absorbed from the gastro-intestinal tract (by subtraction of the faecal amounts after i.v. doses from those after oral doses, see Table 9.8) with the calculated bioavailability, which is the fraction of dose coming into the systemic circulation, suggests that a certain degree of first-pass elimination, about 10-15% of the dose, is present.

Typically, the differences in half-life between the intravenous and oral trial were in general the most pronounced for the hospitalized persons (see Tables 9.3, 9.4, 9.5). Little is known of the effect of continuous bedrest per se on the pharmacokinetic behaviour of drugs. A report by Levy (1967) showed that bedrest alone had a significant influence on the disposition of benzylpenicillin, although the underlying mechanism was not understood. Our data do at least suggest that subjects with bedrest are more susceptible to non-renal increases in elimination rate. These changes could be due to the obviously higher concentrations of chlorthalidone reached initially with intravenous administration, making available higher free drug concentrations, somehow affecting their own elimination. Only in one subject (A.Kl) the shorter t½ after the intravenous dose was concomitant with an increase in renal clearance (Table 9.7), the change thus not being of non-renal character. One may speculate that the underlying hypothyroidism, a serious metabolic disturbance (Werner and Ingbar, 1971), made this subject unable to react upon sudden increases of drug concentration by a non-renal, e.g. metabolic, mechanism and that therefore a secondary change in renal clearance became evident.

Central volume of distribution

The volume of distribution of the central compartment (V1) which was estimated from the intravenous study at a mean value of 36.4 liter (± 10.4 S.D.) or 0.48 liter/kg body wt. (± 0.08 S.D.) (detailed data in Table 9.3) was still larger than the plasma volume, indicating rapid equilibration, with regard to the infusion rate, with well-perfused tissue. A very rapid disappearance of plasma concentrations was evident shortly after the dose in the intravenous study, see Table 9.3 for individual data. This segment can be explained possibly by a strong liver uptake of chlorthalidone, which has been observed immediately after intravenous doses of this drug in rat ex-
periments (Beisenherz et al., 1966). After oral administration no such phase of rapid decay was seen. This is not surprising because the aforementioned decay showed a mean half-life of ca. 20 min, which is of about the same magnitude as the rate of absorption of the drug after oral administration, for which a mean half-life of ca. 19 min was measured (see Table 9.4 for personal data). As a result, much larger apparent central volumes of distribution were found for the oral study, even if corrected for the bioavailability F. Thus, the $V_1/F$ values obtained in the oral study cannot be compared directly with those for $V_1$ from the intravenous trial, because different models were necessary to describe the time course of the plasma concentrations after each dosage form.

**Plasma versus red blood cell half-lives**

The mean half-life in the present seven subjects for the terminal decay phase was, in the intravenous and oral study respectively, 36.5 h ($\pm$ 10.5 S.D.) and 44.1 ($\pm$ 9.5 S.D.) for the plasma concentrations and 46.4 h ($\pm$ 9.9 S.D.), respectively 52.7 h ($\pm$ 9.0 S.D.) for the erythrocyte concentrations. A pharmacokinetic model including non-linear binding of chlorthalidone to red blood cells, which is able to explain in detail the observed half-life differences between plasma and erythrocyte decay, is described extensively in Chapter 7 (Fleuren and van Rossum, 1977). Some comment has to be made yet on the magnitude of the differences in the present study, being somewhat smaller than those previously reported, when an average terminal $t\frac{1}{2}$ of 40 hours for the decay of the plasma concentrations and of 60 hours for that of the erythrocyte concentrations was found after single 100 or 200 mg oral doses in ten human subjects (Chapter 7). A lower dose, mostly 50 mg, was administered in the present experiments, consequently it took less time to reach the low concentrations, at which strong binding of drug to components of the erythrocytes becomes the predominant factor governing the slow transport rate to plasma. There is good evidence that carbonic anhydrase has an important role in this strong binding (Beermann et al., 1975; Dieterle et al., 1976; Chapter 8).

An apparent discrepancy should be noticed in this context between recent pharmacokinetic reports on chlorthalidone by different authors. Our results were, shortly summarized, parallel decays of plasma concentrations and urinary excretion rate, being shorter in half-life than the $t\frac{1}{2}$ of the erythrocyte concentrations during at least 100-200 hours after the dose (Fleuren and van Rossum, 1975, 1977; Chapter 7; this Chapter). In contrast, other authors estimated parallel decays of plasma concentrations, urinary excretion rate and red blood cell concentrations* (Riess et al., 1977), stated that plasma concentrations "mirrored" red blood cell concentrations (Collste et al., 1976) or reported variable figures with relation to the half-life values (Beermann et al., 1975). One explanation for this
divergency can be found in the circumstance that methodological precautions have to be taken for the precise assay of chlorthalidone plasma concentrations, otherwise much too low values will be found, especially during the first 10-24 hours after the dose (Fleuren and van Rossum, 1978; Chapter 3). If these conditions are not fulfilled (Beerman et al., 1975; Collste et al., 1976), or alternatively, if the concentrations during the first 24 hours are not measured (Riess et al., 1977), the higher initial plasma concentrations, which contribute much to establish a faster decay phase, are ignored and a wrong impression of the half-life is obtained.

Additionally, it may be argued that exact half-lives from urinary excretion data are difficult to assess, if only 24 hour portions have been collected (Riess et al., 1977), the more so as considerable fluctuations in urinary clearance from day to day do occur, which can be seen by inspection of e.g. Figure 9.5. Nevertheless, also Riess and co-workers (1977) reported a faster half-life from urinary excretion data than from whole blood concentrations at higher concentrations reached after chronic administration, although they did not comment on this observation. Analogous pharmacokinetics, viz. a slower decay of erythrocyte concentrations than of those in plasma, have been reported for acetazolamide, a drug also strongly bound to carbonic anhydrase in red blood cells (Maren, 1962), in both humans (Wallace et al., 1977) and dogs (Maren, 1962).

It has been suggested in literature that the long biological half-life of chlorthalidone could be maintained by a prolonged absorption of the drug from the gastro-intestinal tract (Pulver et al., 1959). Evidence against this possibility is raised by the present faecal excretion data after oral administration, details of which showed that 80-90% of this non-absorbed fraction was excreted already with the first faeces portion in most subjects, which was commonly produced at the first or second day. It is reasonable to assume that if further considerable amounts of non-absorbed drug would have been present in the gut lumen at later time, this had been reflected also in the faecal excretion during that period. This implies that the absorption had been terminated amply before the end of the period, in which the long $t_{1/2}$ values were evident, viz. up to ten days and longer. Another contribution to a long half-life could theoretically have been caused by enterohepatic circulation, as chlorthalidone was found to be excreted in the bile of rats (Beisenherz et al., 1966). Human experiments, however, revealed the biliary excretion of only a few percent of unchanged drug

* Actually measured in whole blood, but due to much higher concentrations in red blood cells than in plasma, a perfect resemblance between the terminal decay of whole blood and red blood cell concentrations can be assumed.
In conclusion, it must be stated that the long biological half-life of chlorthalidone is caused to an important degree by the strong binding of the drug to red blood cells (Beermann et al., 1975; Fleuren and van Rossum, 1975; Collste et al., 1976; Fleuren and van Rossum, 1977; Riess et al., 1977; This thesis, Chapters 7 and 8), and by the relatively slow exchange rate with that tissue, see e.g. Figures 9.2 and 9.3, together providing very low concentrations of unbound drug available for elimination.

Evidence for metabolic clearance

As the total urinary excretion of unchanged drug after intravenous administration amounted to only 65.4 ± 8.6% of the dose (mean ± S.D.) and only a few percent was found in the faeces (Table 9.8), while an approximately equal cumulative amount in urine plus faeces was recovered after oral administration (Tables 9.7 and 9.8), strong evidence exists for another important, presumably metabolic, route of elimination of chlorthalidone in man. Essentially the same information can be obtained by comparison of the plasma clearance (Table 9.3) with the corresponding renal clearance values (Table 9.7), the latter being on an average 38% lower than the total clearance. In agreement with metabolic degradation of chlorthalidone is the observation that, after oral administration of C14-chlorthalidone to humans, about 10% of the urinary radioactivity showed thin-layer chromatographic behaviour different from that of the original compound (Beermann et al., 1975). Metabolism of C14-chlorthalidone in the rat has been demonstrated by Beisenherz et al. (1966), the greatest part of the metabolites being excreted in the bile. A minor part of this radioactivity was tentatively attributed to the hydrolysis product of chlorthalidone, 3-(4-chloro-3-sulfamoylbenzoyl)-benzoic acid. This compound was not found in human urine and bile (Chapter 11). Thus, the nature of the metabolic pattern of chlorthalidone in man has yet to be identified.

SUMMARY

Seven normal human volunteers received each a constant-rate infusion of chlorthalidone during 2 hours and an equal - commonly 50 mg - single oral dose on separate occasions. Concentrations of unchanged chlorthalidone were analyzed during 100 to 200 hours periods in plasma, red blood cells, urine and faeces after both dosage modes. A three compartment model was necessary to describe the intravenous plasma concentrations in the majority of subjects (five). A two compartment model was suf-
sufficient for the decay of the oral plasma concentrations in all seven subjects.

The mean plasma $t_{1/2}$ after i.v. doses was $36.5 \text{ h (± 10.5 S.D.)}$, the mean plasma $t_{1/2}$ after oral doses was $44.1 \text{ h (± 9.6 S.D.)}$. The mean red blood cell concentration $t_{1/2}$ after i.v. doses was $46.4 \text{ h (± 9.9 S.D.)}$ and the mean red blood cell $t_{1/2}$ after the oral doses was $52.7 \text{ h (± 9.0 S.D.)}$. The urinary excretion rate plots were parallel to the plasma concentration curves in all cases. As the faster decay after i.v. administration was not accompanied by an increased renal clearance, the difference had to be caused by a non-renal mechanism. A mean cumulative amount of $65.4 \text{ (± 8.6 S.D.) \%}$ of the intravenous dose was excreted in urine over infinite time, whereas a mean value of $43.8 \text{ (± 8.5 S.D.) \%}$ was found for the oral dose. Faecal excretion ranged from $1.3\text{-}8.5\%$ of dose in the i.v. study and from $17.5\text{-}31.2\%$ of dose in the oral study. The sum of the amounts present in urine plus faeces strongly pointed to an important metabolic route of elimination of chlorthalidone. Bioavailability estimates ($F$) were obtained from three sets of data: a mean $F = 0.61$ from plasma concentrations, a mean $F = 0.67$ from urinary excretion measurements and a mean $F = 0.72$ from the erythrocyte concentrations. Simulations with a non-linear model indicated the lesser validity of the estimate from erythrocyte concentrations. It was concluded that the average of plasma and urine data, $F = 0.64$, yielded the best estimate of the oral availability of 50 mg chlorthalidone doses in man.

REFERENCES


Wagner, J.G.: Linear pharmacokinetic equations allowing direct calculation of many needed pharmacokinetic parameters from the coefficients and exponents of polyexponential equations which have been fitted to the data. J Pharmacokm Biopharm. 4, 443-467 (1976).


INTRODUCTION

Diuretics play a crucial role in current treatment of mild to moderate hypertension. Several recent studies have compared the antihypertensive effects of different dosage levels of chlorthalidone (Hygroton®), ranging from 12.5-200 mg per os daily (Bengtsson et al., 1975; Carney et al., 1976; Materson et al., 1978; Tweeddale et al., 1977). The general conclusion was that with the 25 mg dose an equal or almost equal blood pressure reduction was achieved as with the larger amounts.

The relationship between the natriuresis, resulting in a reduced extracellular volume, and the antihypertensive effect of diuretics seems to be firmly established (Bennet et al., 1977; de Carvalho et al., 1977). On the other hand, indications exist for a dependence of natriuresis upon the excretion in the urine of the diuretic drug itself, which was demonstrated in the case of chlorothiazide (Beyer and Baer, 1961), hydrochlorothiazide (Beyer and Baer, 1975) and furosemide (Hook and Williamson, 1965; Homeida et al., 1977; Honari et al., 1977) by interaction studies with probenecid. Some evidence for such coupling of natriuresis with urinary excretion rate of chlorthalidone has been presented in Chapter 1.

The above considerations suggested a detailed study of the involvement of pharmacokinetic factors, with emphasis on the urinary excretion parameters, in the clinical dose-response curve of chlorthalidone.

METHODS

Subjects and dosage

Single oral doses of chlorthalidone were administered as commercial tablets (Hygroton®, Ciba-Geigy) to 17 young healthy volunteers (age 20-32 yr, body weight 65-88 kg, 2 females, 15 males). After fasting overnight, the subjects ingested the tablet in the morning at 9.00 a.m., crushed it by the teeth and swallowed it completely with about 200 ml of tap water. No food or beverages other than water were allowed until ca. 4 hours after
dosage. The 50 mg dose was received by 6, the 100 mg dose by 11 and the 200 mg dose by 6 subjects. Three individuals in this group took both the 50, the 100 and the 200 mg tablet in a randomized order, each dose 1 month apart. In addition, 2 subjects received the same dose - 100 mg - twice, on separate occasions, in order to get an impression of possible intra-individual variation in the handling of the drug. Nobody received any other medication at least 2 weeks prior to or during the trial.

The influence of urinary pH upon renal plasma clearance of chlorthalidone was studied in a separate experiment by giving two subjects capsules with sodium bicarbonate or ammonium chloride. From the second day after intake of 100 mg of chlorthalidone, urinary pH was adjusted either at pH 5.2 with NH₄Cl or at pH 7.5-8 with NaHCO₃ by means of hourly pH measurements. Subject T.Ho started with NaHCO₃ for two days (20 g on day 2, 13.5 g on day 3) followed by two days with NH₄Cl (7 g on day 4, 3 g on day 5). Subject M.Br made his urine acidic or alkaline in a different sequence (20 g NaHCO₃ on day 2, 7 g NH₄Cl on day 3, 19.5 g NaHCO₃ on day 4, 8 g NH₄Cl on day 5).

**Biological samples and assay**

Urine was collected in separate or 6-12 hr portions (Chapters 7 and 9, respectively), in most cases during 100 hr after the dose (range 80-220 hr, exact time periods indicated in Table 10.1). Venous blood portions of about 7 ml, spread over the sampling period as indicated in the Chapters 7 and 9, were heparinized and centrifuged immediately after collection in order to ensure correct values of the plasma concentrations in the way described in Chapter 3. The plasma and red blood cell fractions and aliquots of each urine portion were frozen at -20°C until assay. The concentrations of chlorthalidone were determined by gas chromatography with nitrogen detection (Fleuren and van Rossum, 1978; Chapter 3).

**Pharmacokinetic analysis**

In the present between-dose comparison of chlorthalidone data from subjects participating in the pharmacokinetic studies described in the Chapters 7 and 9 were also used. The urinary excretion rate versus time plots were found always to parallel the decay curves of the plasma concentrations of chlorthalidone. On the contrary, the elimination half-lives of the red blood cell concentrations, being 50-100 times higher than those in plasma, were considerably longer than the values obtained from the corresponding plasma concentrations after single 100 or 200 mg doses. A non-linear pharmacokinetic model, proposed in Chapter 7, takes all above features of the time courses of both plasma and red blood cell concentrations into account. Yet, it had to be expected, on the basis of that model, that the decay of plasma concentrations and urinary excretion rate should
ultimately approximate the decay of the red blood cell curves, when concentrations had become sufficiently low. Experiments with single 50 mg doses indicated that the half-life of the urinary excretion rate plots indeed converged to the longer half-life of the erythrocyte concentrations, when measurement was performed over very long time periods (200-336 hr) (Fleuren et al., 1979a; Chapter 9). In view of this information, it was judged reasonable to extrapolate the urinary excretion rate plots to infinite time with the use of the t½ value of the erythrocyte concentrations and so obtain the total amount excreted. An overestimation of this amount will result if the slower decay rate has not yet been reached at the time point where extrapolation actually begins. However, as the difference between the cumulative amounts, calculated by using either the t½ of the red blood cell concentrations or the t½ obtained from the urinary excretion rate plots, is relatively small (maximally ca. 10% in a few cases), the error possibly made herein must be even smaller, because the true value lays between these two extremes. Therefore, this method of extrapolation has a negligible quantitative influence on the total urinary recovery.

The renal clearance of chlorthalidone was calculated by dividing the amount actually excreted in the urine during the assay period by the corresponding area under the plasma concentration curve. The elimination half-lives of plasma and erythrocyte concentrations were determined by non-linear regression analysis of the final straight part of the curves, using at least 5 data points. The areas under the concentration-time curves were obtained by the trapezoidal rule.

RESULTS

Urinary excretion rate of chlorthalidone vs time, plasma and red blood cell concentration decay

A typical example, in which the absorption and distribution of chlorthalidone in both components of blood and its elimination into urine are shown, is given in Figure 10.1. Both a 100 mg and a 200 mg dose of the drug were administered on separate occasions to a healthy human subject. After a rapid absorption into the plasma, with peak concentrations occurring at approximately 2 hours following the dose, the drug was transported relatively slowly into the erythrocytes, where maximum concentrations were not found before 10-15 hours. Red blood cell concentrations, ca. 100 times higher than those in plasma, persisted during the post-distribution phase. The half-life of the erythrocyte concentrations was longer than the plasma half-life (detailed data shown in Table 10.1). The urinary excretion rate was parallel to the time course of the plasma concentrations. So far,
Figure 10.1
Urinary excretion rate, plasma (*) and red blood cell (o) concentration profiles of chlorthalidone following oral administration of a single 100 mg and 200 mg dose to the same subject. In the lower graphs the cumulative urinary excretion during 100 hours is shown, which amounted to 37.0% and 23.2% of the dose after 100 mg and 200 mg respectively. For further explanation see text.

these pharmacokinetic aspects of chlorthalidone were completely consistent with the observations described in the Chapters 7 and 9. The next section deals with the total urinary recovery at different dose levels of the drug.
Cumulative urinary excretion

The total urinary recovery of chlorthalidone is not a linear function of the dose (Fig. 10.2). The percentage of dose excreted in the urine was the same after single doses of 50 and 100 mg, 44.4% and 43.1% respectively, but decreased sharply to 29% after a 200 mg dose. The individual data of 17 subjects are presented in Table 10.1: the amount excreted at infinite time ranged from 16.0-25.4 mg after a 50 mg dose in 6 subjects, from 36.2-52.4 mg after 100 mg in 11 subjects, and from 43.4-66.9 mg after 200 mg in 6 subjects. The individual urinary recoveries of the subjects, who had received three dose levels of the drug (Table 10.1: J.Bi, D.Bo, T.Sw), were entirely consistent with the average pattern of Figure 10.2.

![Cumulative urinary excretion](image)

Figure 10.2
Cumulative amount of chlorthalidone recovered in urine after oral administration of single doses of different magnitude to human healthy subjects. The values shown (mean ± S.D., n = number of experiments) were obtained by extrapolation to infinite time from the actual assay period, which ranged from 80-220 hours. The average percentage of excretion decreased from 44.4% and 43.1% of dose after 50 mg and 100 mg respectively to 29.0% after the 200 mg dose.

Renal clearance

A considerable interindividual variation in the renal plasma clearance of chlorthalidone was observed, ranging from 3.1-7.5 l/hr (52-125 ml/min) after the 50 mg dose, from 2.6-6.4 l/hr (43-107 ml/min) after that of 100 mg and from 2.8-4.6 l/hr (47-77 ml/min) after the dose of 200 mg. Intraindividual variation in this extent can probably be ruled out by comparison
<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Subject</th>
<th>Assay period (hr)</th>
<th>Half-life (hr)</th>
<th>AUC plasma (hr.mg/l)</th>
<th>Renal clearance (l/hr)</th>
<th>Cumulative urinary recovery (mg) during assay</th>
<th>Cumulative urinary recovery (mg) at infinite time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>J.Bi</td>
<td>100</td>
<td>46</td>
<td>46</td>
<td>2.05</td>
<td>6.28</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>D.Bo</td>
<td>100</td>
<td>48</td>
<td>62</td>
<td>2.53</td>
<td>7.51</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>L.De</td>
<td>219</td>
<td>51</td>
<td>60</td>
<td>6.89</td>
<td>3.10</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>T.HoC</td>
<td>195</td>
<td>43</td>
<td>50</td>
<td>6.73</td>
<td>3.60</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>M.Oc</td>
<td>219</td>
<td>48</td>
<td>52</td>
<td>4.58</td>
<td>4.66</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>T.S</td>
<td>100</td>
<td>45</td>
<td>57</td>
<td>3.65</td>
<td>4.44</td>
<td>16.2</td>
</tr>
<tr>
<td>Mean ± S.D. (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.93</td>
<td>± 1.68</td>
</tr>
<tr>
<td>100</td>
<td>J.Bi</td>
<td>80</td>
<td>42</td>
<td>52</td>
<td>4.30</td>
<td>6.33</td>
<td>27.2</td>
</tr>
<tr>
<td></td>
<td>D.Bo</td>
<td>100</td>
<td>50</td>
<td>60</td>
<td>5.75</td>
<td>6.43</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td>M.Br</td>
<td>100</td>
<td>45</td>
<td>66</td>
<td>8.29</td>
<td>3.52</td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>M.Br</td>
<td>200</td>
<td>46</td>
<td>65</td>
<td>14.20</td>
<td>3.34</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>H.v.B</td>
<td>200</td>
<td>45</td>
<td>60</td>
<td>9.14</td>
<td>4.32</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>V.Fl</td>
<td>100</td>
<td>30</td>
<td>51.5</td>
<td>8.19</td>
<td>3.72</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>V.Fl</td>
<td>94</td>
<td>31</td>
<td>54</td>
<td>9.69</td>
<td>3.38</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>P.K</td>
<td>100</td>
<td>32</td>
<td>59</td>
<td>12.80</td>
<td>2.78</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>O.L</td>
<td>100</td>
<td>39</td>
<td>62</td>
<td>10.70</td>
<td>2.98</td>
<td>31.9</td>
</tr>
<tr>
<td></td>
<td>M.Pd</td>
<td>100</td>
<td>34</td>
<td>54</td>
<td>14.30</td>
<td>2.56</td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td>M.R</td>
<td>100</td>
<td>42</td>
<td>68</td>
<td>9.99</td>
<td>3.71</td>
<td>37.1</td>
</tr>
<tr>
<td></td>
<td>T.S</td>
<td>100</td>
<td>55</td>
<td>69</td>
<td>6.45</td>
<td>4.84</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>L.V</td>
<td>96</td>
<td>29</td>
<td>52</td>
<td>8.01</td>
<td>4.28</td>
<td>34.3</td>
</tr>
<tr>
<td>Mean ± S.D. (n = 13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.02</td>
<td>± 1.23</td>
</tr>
<tr>
<td>Subject</td>
<td>Period after dose (hr)</td>
<td>Average urinary pH</td>
<td>Average urinary flow (ml/hr)</td>
<td>Average renal clearance (l/hr)</td>
<td>Period after dose (hr)</td>
<td>Average urinary pH</td>
<td>Average urinary flow (ml/hr)</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
<td>--------------------</td>
<td>----------------------------</td>
<td>----------------------------</td>
<td>------------------------</td>
<td>--------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>J.Bi</td>
<td>25-37</td>
<td>7.5</td>
<td>249</td>
<td>5.3</td>
<td>25-39</td>
<td>7.6</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>50-71</td>
<td>5.2</td>
<td>85</td>
<td>4.9</td>
<td>47-65</td>
<td>7.8</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>73-95</td>
<td>7.5</td>
<td>132</td>
<td>5.4</td>
<td>74-88</td>
<td>5.2</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>96-110</td>
<td>5.1</td>
<td>123</td>
<td>4.0</td>
<td>95-108</td>
<td>5.1</td>
<td>69</td>
</tr>
</tbody>
</table>

TABLE 10.1 continued

<table>
<thead>
<tr>
<th>Subject</th>
<th>Period after dose (η = 6)</th>
<th>Mean ± S.D. (n = 6)</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.Bi</td>
<td>100</td>
<td>36 52</td>
<td>100</td>
<td>10.00</td>
<td>4.46</td>
<td>43.4</td>
</tr>
<tr>
<td>D.Bo</td>
<td>100</td>
<td>42 64</td>
<td>100</td>
<td>13.50</td>
<td>3.46</td>
<td>62.4</td>
</tr>
<tr>
<td>H.F</td>
<td>80</td>
<td>34 58</td>
<td>80</td>
<td>22.50</td>
<td>2.81</td>
<td>64.4</td>
</tr>
<tr>
<td>O.K</td>
<td>185</td>
<td>43 60</td>
<td>185</td>
<td>43</td>
<td>63.3</td>
<td>66.9</td>
</tr>
<tr>
<td>J.Med</td>
<td>168</td>
<td>33 45</td>
<td>168</td>
<td>10.90</td>
<td>4.31</td>
<td>48.3</td>
</tr>
<tr>
<td>T.S</td>
<td>100</td>
<td>42 70</td>
<td>100</td>
<td>9.78</td>
<td>4.26</td>
<td>62.6</td>
</tr>
</tbody>
</table>

a The urine t½ was equal to the plasma t½
b The extrapolation to infinity was performed with the use of the t½ of the erythrocyte concentrations. For further explanation, see methods section
c The subjects T.Ho and M.O received 50.4 and 60.9 mg respectively (Chapter 9). Their AUC and urinary recoveries have been standardized to the 50 mg dose level by multiplying the original data with 5/5.04 and 5/6.09 respectively
d Subjects of female sex

TABLE 10.3
Effects of urinary pH and flow on renal plasma clearance of chlorthalidone
of the average renal clearance values in two subjects (M.Br and V.Fl), who had received the same dose twice at different occasions (Table 10.1). A firm negative correlation was found between the renal plasma clearance and the area under the plasma concentration curve* (correlation coefficient $R = -0.86$, significantly different from zero at $P < 0.005$), whereas there was no significant correlation between the renal plasma clearance and the total urinary recovery ($R = 0.11$).

The data of the subjects ($n = 3$), who received each both the 50, 100 and 200 mg doses are presented separately in Table 10.2. While the renal clearance in one subject did not change with dose systematically, but showed variations of the same order as those in subjects administered an equal dose twice** (intraindividual variation), the clearance in the other two subjects decreased as the doses increased from 50 to 200 mg. The mean value ($n = 3$) after 200 mg (74 ml/min) was 27% lower than that after 50 mg (101 ml/min). Figure 10.3 represents the urinary excretion rate plotted against plasma concentrations of chlorthalidone, measured during 100 hr after administration of two different doses to the same human subject. The picture demonstrates that the difference in the slope of both plots, viz. in the magnitude of the renal clearance, remained unchanged during the entire experimental period.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Renal clearance (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mg</td>
</tr>
<tr>
<td>J.Bi</td>
<td>105</td>
</tr>
<tr>
<td>D.Bo</td>
<td>127</td>
</tr>
<tr>
<td>T.S</td>
<td>74</td>
</tr>
<tr>
<td>Mean ($n = 3$)</td>
<td>101</td>
</tr>
</tbody>
</table>

* Calculated from experiments with an equal sampling period (100 hr, $n = 8$, dose 100 mg, see Table 10.1).

** cf. Table 10.1: subjects M.Br and V.Fl.
Figure 10.3
Two plots of the urinary excretion rate of chlorthalidone against the plasma concentration at the midpoints of the urine collection periods, measured during 100 hours after oral administration of varying single doses at different occasions to the same human subject. A diminution of the renal plasma clearance, $k_{CR}$, from 107 ml/min to 77 ml/min was observed when the dose was increased from 100 mg to 200 mg.

Dependence of renal clearance upon urinary pH and flow
The urinary excretion rate of chlorthalidone is subject to large fluctuations. Because the decay of plasma concentrations of the drug against time is very regular, the profiles of plots of urinary excretion rate and renal plasma clearance are almost identical (Fig. 10.4). Table 10.3 shows the average renal plasma clearance of chlorthalidone under conditions of acidified or alkalinized urine production by two human subjects. The time-averaged clearance values appeared to depend only slightly upon urinary pH and not upon urine flow. Although some coincidence could be observed between incidental changes in flow and renal clearance for urine kept at constant pH, the overall pattern of excretion appeared to be governed by other factors. Large variations were visible which did not cor-
Figure 10.4
Plots of plasma concentration and urinary excretion rate of chlorthalidone against time, revealing large fluctuations in renal plasma clearance. The time-averaged renal clearance, indicated by dotted lines in the lower graph, did increase only slightly when urine pH was changed from 5.2 to 7.7. Black horizontal bars at the bottom indicate periods of bed rest. During these periods a striking decrease in renal clearance compared to that by day became apparent.
respond to changes in flow or urinary pH (Fig. 10.4). This held true also for all subjects not receiving ammonium chloride or sodium bicarbonate, from whom urine had been obtained in separate portions. By sampling urine over longer periods, e.g. 12-24 hr, excessive fluctuations became masked (cf., for instance, Fig. 9.6). Part of the fluctuations occurred in a circadian rhythm: renal clearance at night was always decreased compared to the clearance at day. This was the case in every subject studied (see e.g. Fig. 10.4).

DISCUSSION

**Dose-dependent urinary excretion**

Several factors can be responsible for the relatively small increase in urinary recovery of chlorthalidone on changing the oral dose from 100 to 200 mg: limited absorption from the gastro-intestinal tract, decreased renal clearance or a combination of both. No definite evidence for only one of these possibilities is available so far.

In Chapter 9, we reported an absolute bioavailability of only 64%, on an average, after oral administration of a single 50 mg dose to 7 human subjects. Because this incomplete bioavailability was mainly attributable to the amount of unabsorbed drug, a progressively decreasing percentage of absorption at higher oral doses would be conceivable. To clarify this point, we measured, as described in Chapter 9, concentrations of unchanged chlorthalidone in the faeces of two subjects participating in the present 200 mg dose study. Over 7 days, 21% and 17% of the dose were found (for the subjects O.K. and J.Me, respectively). These data practically represent the unabsorbed fraction of the drug, because biliary excretion of chlorthalidone in man is very small (Fleuren et al., 1979b; Chapter 11). Therefore, compared with the single 50 mg dose, of which 17.5-31.2% was recovered in faeces after oral administration (Chapter 9), no disproportional change in the fraction of unabsorbed chlorthalidone has become evident after the 200 mg dose.

The interindividual variation in the renal plasma clearance of chlorthalidone is in the same order as the differences found by Collste et al. (1976) after single 50 mg doses. The large standard deviations (Table 10.1) did not permit a conclusion with regard to the possible influence of dose on this parameter. However, in two of the three subjects in whom a clear comparison of three doses could be made, there was a decrease in renal clearance after the 200 mg dose, see Table 10.2. A similar conclusion on this matter can be deduced from data published by Riess and coworkers (1977), who found a ratio of 1:2:2.6 for the cumulative amounts of chlor-
thalidone excreted in urine after single 50 mg, 100 mg and 200 mg doses in the same 6 subjects, whereas the ratio of the areas under the curve of the corresponding whole blood concentrations was 1:2.3:4. Also for the diuretic bendroflumethiazide a lesser renal clearance has been reported after a single 5 mg dose compared to that after a single 2.5 mg dose in hypertensive patients (Beermann et al., 1978).

Renal tubular secretion is an important pathway for the elimination of sulfonamide-type diuretics (Beyer and Baer, 1961, 1975; Chapter 1). The parallel decay of plasma concentrations and urinary excretion rate plots of chlorthalidone (Fleuren and van Rossum, 1975; Fleuren et al., 1977, 1979a; Chapters 7, 9 and 10) does not support the hypothesis of a simple concentration-dependent decrease in tubular secretion after higher doses of drug. This can also be seen from Figure 10.3 where completely different renal clearance values were obtained after two dosage levels, while the range of plasma concentrations was virtually the same. If tubular secretion function should be involved, it must have changed in a non-competitive manner, because the different status of the kidney with respect to clearance of the diuretic remains strikingly constant during a rather long period (Fig. 10.3).

**Fluctuations in renal plasma clearance**

The time-averaged renal plasma clearance of chlorthalidone was not susceptible to changes in urine flow (in the range 70-250 ml/hr). This confirmed similar findings described in Chapter 9. The renal clearance was influenced by urinary pH but the effect was small. This does not need to surprise, however, because the pKₐ of chlorthalidone is 9.35 (Fleuren et al., 1979c; Chapter 6), which implies that the percentage of ionized drug increases from 0.01% at pH 5.3 to only 3.5% at pH 7.8. Beyer and Baer (1975) reported an about equally small rise in renal plasma clearance of chlorthalidone with increasing pH in the dog. Nevertheless, as is discussed in Chapter 1, the pH effect indicates that also tubular reabsorption determines besides secretion the net excretion of this drug into the urine.

Many endogenous substances are actively secreted and/or reabsorbed by the kidney. Among these are metabolic substrates which provide the energy for the tubular reabsorption of salts and water. Proximal tubular cells do not utilize glucose as their main energy source, but prefer lactate, α-ketoglutarate and free fatty acids (Cohen and Barac-Nieto, 1973). These acidic substrates are known to compete for uptake in the renal cortex with sulfonamide diuretics and other drugs secreted by the renal organic acid transport system (Cohen and Barac-Nieto, 1973). Accumulation in proximal tubules has been established also for chlorthalidone (Taugner and Iravani, 1965). So, it is reasonable to assume that diuretics and endogenous substrates utilize a common secretory pathway. The large varia-
tions in renal clearance of chlorthalidone (Fig. 10.4) could then result from mutual competition for secretion. Analogously, competition for tubular reabsorption is conceivable for instance with uric acid which is handled both by active secretion and reabsorption in the kidney (Mudge et al., 1973). Further studies are required to know which of these endogenous compounds or other substances (e.g. food constituents) do significantly influence the persistence of diuretics in renal tissue. The circadian rhythm in the renal plasma clearance of chlorthalidone might well be related to one or more of the many physiological changes known to occur in the kidney at night, e.g. the reduction of the glomerular filtration rate (Mertz, 1976). This issue too remains to be investigated.

**Therapeutic meaning**

Our investigations showed a small increase in the cumulative urinary excretion of chlorthalidone when the dose was changed from 100 to 200 mg. In view of the long biological half-life of the drug, see e.g. Table 10.1, the concentrations reached after repetitive lower doses, e.g. 50 mg/day, can be expected to approach those encountered after administration of a single 200 mg dose. In that case, the pharmacokinetic limitations described here may well be related to the limited or nihil gain in the therapeutic response of higher chronic doses, reported recently (Bengtsson et al., 1975; Carney et al., 1976; Materson et al., 1978; Tweeddale et al., 1977). The relationship between the natriuretic and antihypertensive effect of diuretics and their own urinary excretion after chronic administration still seems an unsolved problem, worthy of further investigation.

**SUMMARY**

Renal excretion, plasma concentration and erythrocyte concentration profiles of chlorthalidone were studied in 17 healthy human subjects during 100-200 hours after oral administration of single doses of 50, 100 and 200 mg. Three subjects received all three doses in a cross-over design. The other 14 were involved in the study at one dosage level. The 50 mg dose was taken altogether by 6, the 100 mg dose by 11 and the 200 mg dose by 6 subjects.

The effects of urinary pH and flow upon renal clearance of chlorthalidone were explicitly studied in two subjects in a separate experiment. The renal clearance varied considerably over time and the fluctuations were not attributable to changes in urinary pH or flow.

Total urinary recovery of chlorthalidone extrapolated to infinity was 44.4% of the 50 mg dose (22.2 ± 3.4 mg), 43.1% of the 100 mg dose (43.1 ± 4.7 mg), but only 29.0% of the 200 mg dose (58.0 ± 9.7 mg; mean
values ± S.D.). A large interindividual variation in the renal plasma clearance of chlorthalidone was observed, 52-125 ml/min after 50 mg, 43-107 ml/min after 100 mg and 47-77 ml/min after 200 mg. This variation correlated more with differences between subjects in the area under the plasma concentration curve than with those in total urinary recovery of the drug. The subjects who were available for a between-dose comparison of the renal clearance showed an average decrease in it of 27%, in going from the single 50 mg to the 200 mg dose, suggesting a causal relationship with the limited urinary recovery of the drug at the higher dose.

REFERENCES


BILIARY EXCRETION OF CHLORTHALIDONE IN HUMANS

INTRODUCTION

In man, chlorthalidone (Hygroton®) is eliminated for the greater part by excretion into urine in unchanged form (Chapters 9 and 10; Fleuren et al., 1979). Following oral administration, urinary recovery measurements alone are not sufficient to prove an additional route of elimination, because the missing part of the dose could have resulted from incomplete absorption in the gastro-intestinal tract. However, after intravenous doses of 50 mg of chlorthalidone, only a mean total of 65% of the dose was recovered in urine (Chapter 9), so that elimination via the liver, either by biliary excretion or by metabolism, should account for the remainder 35%. When Cl4-chlorthalidone was given to rats, radioactivity concentrated not only in the kidneys but also in the liver, which was followed by excretion of label into the bile (Beisenherz et al., 1966). The present study was performed to elucidate biliary excretion of chlorthalidone in man. The drug was given orally to cholecystectomized patients with normal (or nearly normal) liver function. T-tube drainage was used to collect bile from the common bile duct, and chlorthalidone concentrations in bile and urine were analysed.

EXPERIMENTAL PROCEDURE

Patients

Four female and two male subjects gave informed consent for this study. All patients had undergone cholecystectomy and choledochotomy because of choledolithiasis*. Liver and renal function had been judged by routine laboratory tests (S.G.O.T., S.G.P.T., bilirubin, alkaline phosphatase, L.D.H., total protein, hemoglobin, serum electrolytes, plasma creatinine and ureum). Detailed patient data are shown in Table 11.1.

The study started 3 to 4 days postoperatively, when the patients had

* Operations performed at the Department of Surgery, Radboud hospital, Nijmegen, The Netherlands.
}| subject | age (yr) | body weight (kg) | creatinine plasma (μmol/l) | ureum plasma (μmol/l) | total bilirubin (μmol/l) | sGOT (U/l) | sGPT (U/l) | alkaline fosfatase (U/l) | LDH (U/l) | diagnosis of liver function |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M.A.</td>
<td>59</td>
<td>61</td>
<td>77</td>
<td>5.73</td>
<td>&lt;10</td>
<td>10</td>
<td>4</td>
<td>86</td>
<td>—</td>
<td>normal</td>
</tr>
<tr>
<td>A.E.</td>
<td>61</td>
<td>82.5</td>
<td>90</td>
<td>4.92</td>
<td>&lt;10</td>
<td>10</td>
<td>8</td>
<td>86</td>
<td>128</td>
<td>normal</td>
</tr>
<tr>
<td>E.K.</td>
<td>42</td>
<td>57</td>
<td>71</td>
<td>4.65</td>
<td>&lt;10</td>
<td>8</td>
<td>8</td>
<td>79</td>
<td>136</td>
<td>normal</td>
</tr>
<tr>
<td>A.L.</td>
<td>60</td>
<td>56</td>
<td>66</td>
<td>4.21</td>
<td>&lt;10</td>
<td>11</td>
<td>6</td>
<td>78</td>
<td>—</td>
<td>normal</td>
</tr>
<tr>
<td>M.M.</td>
<td>28</td>
<td>54</td>
<td>76</td>
<td>5.62</td>
<td>&lt;10</td>
<td>30</td>
<td>41</td>
<td>122</td>
<td>—</td>
<td>slightly disturbed</td>
</tr>
<tr>
<td>R.V.</td>
<td>49</td>
<td>61</td>
<td>79</td>
<td>5.43</td>
<td>&lt;10</td>
<td>14</td>
<td>16</td>
<td>220</td>
<td>—</td>
<td>almost normal</td>
</tr>
<tr>
<td>normal values</td>
<td>—</td>
<td>—</td>
<td>60-100</td>
<td>3.0-6.0</td>
<td>&lt;10</td>
<td>&lt;15</td>
<td>&lt;15</td>
<td>&lt;120</td>
<td>&lt;175</td>
<td></td>
</tr>
</tbody>
</table>

a. Subjects M.A. and A.E. were males, the others females.
b. Determined pre-operatively; all patients were normal in their plasma albumin, total protein, hemoglobin, electrolytes and E.C.G. pattern.
again a normal food and fluid intake. Each subject received a single oral
dose of 100 or 200 mg of chlorthalidone (Hygroton®, Ciba-Geigy, Basle,
Switzerland), as indicated in Table 11.2, after an overnight fast. Food or
fluid, except water, were not given until about 4 hours after the tablet. No
co-medication was used during the trial. Urine and bile were collected in
24-hours portions during 3 to 7 days after the dose (exact sampling periods
given in Table 11.2. Bile was obtained by gravity drainage, via a T-tube, of
the common bile duct.

Chlorthalidone assay

The volumes of the urine and bile samples were measured and an aliquot
of each portion was kept frozen until determination, at -20°C. The concen-
tration of unchanged chlorthalidone in urine was assayed as described in
Chapter 3. For analysis of bile the following minor modification was found
suitable: 0.1 ml of a well-homogenized bile sample was added to 2 ml of
buffer of pH 9.0 (0.1 M, citrate-phosphate-borate, Teorell and Stenhagen),
containing the internal standard, and extracted twice with 10 ml of
methylisobutylketone-ethylalcohol (MIBK:ALC 98:2, v/v). The combined
organic layers were washed twice with 2 ml of the above buffer, transferred
into a new tube, and re-extracted with 2 ml of 0.1 M aqueous NaOH. In
the alkylation reaction and following steps the alkaline extract was treated
identically as described in the original procedure (Chapter 3). Calibration
graphs were prepared by the use of blank bile, obtained from each patient
prior to administration of the drug. They proved to be linear and to pass
through the origin, while the extraction recovery was the same as for an
equal volume of buffer without bile. Standards of chlorthalidone, in the
10-250 ng/sample range, were used for each analysis of unknown samples.

Bile from each patient was treated with β-D-glucuronidase from
Escheria coli (strain K12, Sigma, St.Louis, Mo., USA) by incubating 0.1
ml, mixed with 1 ml of 0.067 M phosphate buffer pH 6.5 (Sörensen), with
1000 Fishman units of enzym during 16 hours at 37°C. Analogously, β-D-
glucuronidase with arylsulfatase activity from Helix pomatia (2000
Fishman units, same manufacturer) was incubated with 1 ml of 0.1 M
acetate buffer pH 5.2 (Walpole), containing 0.1 ml of bile. Afterwards the
pH was brought to pH 9 with a few drops of 5 M aqueous sodium hydrox­
ide, and the sample was analysed for chlorthalidone as described above.

In order to detect in bile and urine the chemical hydrolysis product of
chlorthalidone, 3-(4-chloro-3-sulfamoyl-benzoyl)-benzoic acid (compound
A), which has been tentatively indicated as a metabolite of chlorthalidone
in rats (Beisenherz et al., 1966), bile and urine samples (0.1 ml
and 1 ml, respectively) were extracted at pH 2, both directly and after
treatment with glucuronidase/sulfatase. The conditions of incubation were similar to those described above, except for omittance of acetate buffer because the acetate anion, extracted as acid at pH 2, interfered with the extractive alkylation reaction. Instead, the pH was adjusted to pH 5.2 with dilute HCl and checked after the incubation. A good separation of compound A and chlorthalidone on the gas chromatographic column (3% SE 30) was obtained by lowering the oven temperature to 240°C. The other conditions of analysis, as described in Chapter 3, remained unchanged. The methylated derivatives of the internal standard, compound A and chlorthalidone eluted at retention times of 3.7, 4.8 and 6.2 minutes, respectively.

RESULTS

Chlorthalidone is excreted into human bile. Figure 11.1 shows concentrations of unchanged drug in 24-hours bile from six cholecystectomy patients, followed during 3 to 7 days after oral administration of single 100 or 200 mg doses. Biliary concentration was 11-44 times smaller than that in urine, on a subject-averaged basis. The values depicted in Figure 11.1 represent in fact amounts excreted per day, when a liver bile flow of 1000 ml/24 hr is assumed (see section Discussion). Thus, the biliary excretion rate was seen to be a small fraction of the urinary excretion rate in each subject and the total biliary recovery of chlorthalidone ranged from 0.6-1.4% of the dose, see Table 11.2. When the amounts in bile and urine were compared directly, from equal sampling periods, 2.5-4.7% and 2.5-5.7% of the quantities found in urine were excreted into bile after doses of 100 and 200 mg of chlorthalidone, respectively (Table 11.2). Glucuronidase and sulfatase treatment of the individual bile samples did not increase chlorthalidone concentrations. Measurable amounts (> 5 ng/sample) of 3-(4-chloro-3-sulfamoylbenzoyl)-benzoic acid as a human metabolite of chlorthalidone in bile or urine were not found.

DISCUSSION AND CONCLUSIONS

Only part of the liver bile can be collected by drainage of the common bile duct. The remainder is lost into the duodenum via the second way of the T-tube. Therefore, the volume of bile recovered indicates a minimum only for the actual bile production by the liver. Liver bile flow in healthy humans may vary from 250-1200 ml/day, with an average of 800 ml/day.
Figure 11.1
Concentration in bile and urinary excretion rate of chlorthalidone in 6 cholecystectomized patients, following an oral dose of 100 or 200 mg. Concentration in bile is numerically equal to biliary excretion rate at a bile flow of 11/l/day. The lower graph shows daily volumes of actually collected bile, by means of T-tube drainage of the common bile duct.

(see, e.g., Rein, 1971). The present cholecystectomized patients were selected on the basis of a normal or almost normal liver function, thereby excluding cholestasis of significant extent (Sherlock, 1975). By the T-tube drainage, however, parts of the bile pool were withdrawn from these subjects. It is well-known that any interruption of the normal enterohepatic circulation of bile acids leads to a marked reduction in total liver bile flow (Wheeler, 1975a), so that our patients most probably were different in this respect from normal healthy subjects. On the other hand, many examples of active biliary secretion of organic compounds have been found (Schanker, 1968), implying that the excretion of such drugs is not necessarily dependent upon bile flow.

In fact, Pratt and Aikawa (1962) reported that the total biliary recovery of the related sulfonamide-diuretic hydrochlorothiazide did not increase above controls, when bile flow was stimulated by secretin or by brom-sulphalein, in dogs. If an analogous situation would apply to chlorthalidone, the biliary concentrations encountered in patients undergoing cholecystectomy and subsequent T-tube drainage, should be even higher
TABLE 11.2
Cumulative excretion of chlorthalidone into bile and urine of patients undergoing common bile duct drainage after cholecystectomy and choledochotomy.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dose (mg)</th>
<th>Assay period (days)</th>
<th>Urinary recovery (mg)</th>
<th>Estimated biliary recovery&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>urine</td>
<td>bile</td>
<td>amount (mg)</td>
</tr>
<tr>
<td>M.A.</td>
<td>100</td>
<td>4</td>
<td>4</td>
<td>24.6</td>
</tr>
<tr>
<td>A.E.</td>
<td>100</td>
<td>5</td>
<td>5</td>
<td>31.6</td>
</tr>
<tr>
<td>E.K.</td>
<td>100</td>
<td>4</td>
<td>4</td>
<td>36.5</td>
</tr>
<tr>
<td>A.L.</td>
<td>200</td>
<td>7.5</td>
<td>3</td>
<td>62.8</td>
</tr>
<tr>
<td>M.M.</td>
<td>200</td>
<td>6</td>
<td>6</td>
<td>46.3</td>
</tr>
<tr>
<td>R.V.</td>
<td>200</td>
<td>7</td>
<td>7</td>
<td>58.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Under assumption of a bile flow of 1000 ml/day

<sup>b</sup> Based upon time period during which both bile and urine had been collected.

than those in healthy subjects, because the excreted amounts concentrated in smaller volumes of bile. Therefore, by assuming a bile flow of 1000 ml/24 hr (see Table 11.1), the estimated cumulative amounts are to be considered as maximum values. It must be concluded that biliary excretion is not a preferred route of elimination of chlorthalidone in man, accounting for only a few percent of the dose. These quantities are consistent with the 1.5-8% of the dose found in the faeces of humans after intravenous administration of chlorthalidone (Chapter 9), indicating that this amount did result indeed from excretion into the bile.

The extent of biliary excretion of chlorthalidone appears to be in the same order as that known from other sulfonamide-type diuretics. Although more than 40% of an i.v. dose of C<sup>14</sup>-labelled chlorothiazide was excreted into bile in the nephrectomized dog, this percentage diminished to almost zero under normal physiological conditions (Baer et al., 1959). From a patient, receiving C<sup>14</sup>-chlorothiazide and T<sup>3</sup>-hydrochlorothiazide simultaneously, only 4.4% and 2.4% respectively, of the radioactivity of both drugs was recovered in the bile during 6 days (Calesnick et al., 1961). This figure corresponds with the total recovery of hydrochlorothiazide C<sup>14</sup>-label in human faeces after intravenous administration, which amounted to 1 and 4% of the dose in two healthy subjects (Beermann et al., 1976).
Biliary excretion by active transport has been demonstrated by Hart and Schanker (1966) for chlorothiazide in dogs. Preliminary evidence for such a process can be gained from a high bile/plasma concentration ratio of unbound drug. Although the plasma concentrations of chlorthalidone were not determined in the present study, they can be estimated indirectly from urinary excretion data, because the relationship between both parameters is known: in our investigation reported in the Chapters 9 and 10 and in that of Riess et al. (1977) a renal plasma clearance of ca. 50-100 ml/min was measured for chlorthalidone. By means of the equation \[ \frac{dQ_r}{dt} = k_{Cr}.C_{pl} \] (where \( \frac{dQ_r}{dt} \) denotes urinary excretion rate, \( k_{Cr} \) renal plasma clearance, and \( C_{pl} \) plasma concentration), plasma concentrations of approximately 0.07-0.14 mg/l are found at an urinary excretion rate of 10 mg/24 hr (which can be considered as a typical value, see Figure 11.1.). After correction for a protein binding of 75% (Dieterle et al., 1976; Collste et al., 1976), free drug concentrations in plasma are estimated at roughly 0.02-0.04 mg/l. The corresponding concentrations of chlorthalidone in bile are clearly much higher, see Fig.11.1. Because human bile contains almost no protein (Wheeler, 1975 b), total drug concentration in it can be assumed to be closely equal to the concentration of unbound drug. A free drug concentration ratio of bile: plasma = 20:1 approximately, can then be deduced by inspection of Figure 11.1. While the demonstration of this concentration gradient alone is not sufficient as proof, it may well serve as first evidence for active secretion of chlorthalidone in bile.

The total recovery of chlorthalidone in urine in the present group of patients (Table 11.1) lays in the same order as the values we found in Chapter 10 after single 100 and 200 mg doses in healthy volunteers. This indicates a similar disposition of the drug in both groups. Doses of different magnitude, 100 and 200 mg, have been employed in this investigation, because of dose-dependence in the renal excretion of chlorthalidone: after oral administration of single 50 and 100 mg doses an average fraction of 44 and 43% of dose, respectively, was recovered in human urine, whereas this figure decreased to 29% after the 200 mg doses. The available evidence pointed to a decrease in renal clearance, rather than a limited absorption at the higher dose level (Chapter 10). These findings had left open the possibility of a compensatory biliary pathway at higher concentration. However, no disproportional increase in biliary amount of chlorthalidone after the 200 mg doses became evident (Table 11.2). This might implicate a greater participation of metabolism in the elimination of such doses. After the smaller doses of chlorthalidone, usually 50 mg, employed in Chapter 9, metabolic clearance has been estimated by us as responsible for removal of ca. 30% of the drug.
A single oral dose of the diuretic chlorthalidone (100 or 200 mg) was given to six cholecystectomized patients with T-tube drainage of the common bile duct, and the 24 hours bile and urine were collected during 3-7 days. Urinary recovery of chlorthalidone was 23-37% of the dose, which is in the range of that in healthy volunteers. Chlorthalidone concentration in bile was 11-44 times lower than urinary concentration in corresponding periods, and biliary recovery was only 0.6-1.4% of the dose. The same relative amount of drug was found in bile, whether the 100 or 200 mg dose had been given (viz., a fraction of 2.5-4.7% and 2.5-5.7% of corresponding urinary amounts respectively). It was concluded that excretion into bile constitutes only a minor route of elimination for unchanged chlorthalidone. Bile samples treated with glucuronidase and sulfatase showed no increase of chlorthalidone concentration. The open acid analogue of chlorthalidone, 3-(4-chloro-3-sulphamoylbenzoyl)-benzoic acid, was apparently not formed as a human metabolite, as evidenced by gas chromatographic analysis of both urine and bile.

REFERENCES


SALIVA CONCENTRATIONS OF CHLORTHALIDONE

INTRODUCTION

A study of the relationship between plasma and saliva concentrations of chlorthalidone was considered worthwhile, because saliva has been suggested as a satisfactory alternative for routine determination of pharmacokinetic parameters (Dvorchik and Vesell, 1976; Graham and Rowland, 1972; Groth et al., 1974; Koysooko et al., 1974; Horning et al., 1977). Use of saliva as the sampling fluid precludes the discomfort of venipunctures, while still information would be provided for the adjustment of dosage regimens. Moreover, saliva concentration was suggested to be closely related to free drug concentration in plasma and might, therefore, show a better correlation with therapeutic effects than total plasma concentration. A comprehensive review of the literature on saliva levels of some 30 drugs was given by Danhof and Breimer (1978). They concluded that good correlations between plasma and saliva concentrations had been reported for only a few drugs (e.g., for phenytoin, primidone, carbamazepine and antipyrine), mostly during chronic medication. In single dose studies, very often irregular saliva over plasma concentration ratios (S/P ratios) had been found. This variability was explicable in part by changes in the pH of saliva, which can rise from 6.5 for resting saliva to 7.4 by stimulation of salivary flow, causing differences in the degree of ionization of weakly acidic or basic drugs. Killmann and Thaysen (1955) and Grüneisen and Witzgall (1974), using stimulated saliva, reported very nice correlations between the pKₐ values and saliva over plasma water concentration ratios of a series of closely related sulfonamide antibiotics. The distribution between saliva and plasma could fully be explained here according to pH-partitioning theory only. However, Feller and Le Petit (1977), employing several drugs of different chemical classes, demonstrated that also lipid solubility and molecular size determined, as could be expected, the rate of transport into saliva, which they deduced from differences in the S/P ratios between resting and stimulated saliva. In addition, also active transport processes have been proposed for some drugs, e.g. penicillin, to operate in the salivary glands (see the review by Danhof and Breimer, 1978).

In this chapter, our studies on the excretion of the diuretic chlor-
thalidone into saliva are described. A single dose of chlorthalidone was administered to three healthy human subjects by the intravenous route, so that contamination of saliva, which forms a possible risk in oral administration studies, was avoided, and plasma and saliva concentrations of the drug were followed for a 100 hours period.

METHODS

Three healthy young males, aging 24-28 yr, weighing 73-83 kg, were asked to collect mixed saliva (spit) into glass vials at regular times (indicated in Table 12.1) during and after a 2 hr-infusion of a chlorthalidone solution, which contained 50.0, 51.5 and 61.3 mg of the drug for L.De, T.Ho and M.O. respectively. The study was performed during participation of the subjects in a bioavailability experiment with chlorthalidone, described in Chapter 9 (Fleuren et al., 1979), where the exact times of blood sampling can be found. No artificial means of saliva stimulation was applied. The volumes of the saliva samples ranged from ca. 2-8.5 ml, with an average of 4.2 ml for L.De, 2.8 ml for T.Ho, and 4.1 ml for M.O.; the mean saliva pH was 6.65 for L.De, 7.21 for T.Ho and 6.77 for M.O. (Detailed data in Table 12.1). Saliva samples were frozen at -20° C until assay.

Chlorthalidone concentration in saliva was assayed by gas chromatography with nitrogen detection, as described in Chapter 3 for the determination of plasma concentration (Fleuren and van Rossum, 1978), with small adaptations. After centrifugation, to remove cellular debris, 2-4 ml portions of saliva were extracted twice at pH 7.4. Almost no background signal was found in the gas chromatograms of blank saliva specimens, so that determinations down to ca. 1-2 ng/ml could be performed using 4 ml portions. Extraction recovery (95%) and reproducibility (5% S.D.) were the same as observed when using equal volumes of buffer solution alone.

RESULTS AND DISCUSSION

The time courses of saliva (S) and plasma (P) concentrations of chlorthalidone are compared in Fig. 12.1. Although a rough parallellity was apparent in the terminal parts of the plots, there was a very irregular S/P ratio over the whole period (Table 12.1). The low S/P ratios observed in the three subjects at ca. 1 hr after the start of the chlorthalidone infusion may be attributed to incomplete equilibration between plasma and saliva,
Figure 12.1

Plasma and saliva concentration profiles of chlorthalidone, followed for ca. 100 hours after a single intravenous dose to three healthy human subjects. Note the considerable fluctuations in saliva concentration, causing large variability in the saliva over plasma concentration ratios.

but no definite explanation can be given for the remainder of the variations. It is not likely that these originated from analytical errors, because the standard deviations of the plasma and saliva determinations would allow a possible error in this ratio of only 10%. There was no correlation between the S/P ratios and either the volume or the pH of saliva collected for any of the subjects. This observation on pH was not unexpected, because the pKₐ of chlorthalidone is 9.35 (Chapter 6), so that pH changes around pH 7, which were very small besides (Table 12.1), barely influence the ionization degree of the drug.

The protein-binding of chlorthalidone in human plasma is ca. 75% (Collste et al., 1976; Dieterle et al., 1976), without great differences between individuals in the extent of binding (range 73-76%, Collste et al., 1976). Moreover, percentual protein-binding is the same over the 0.02-7.7
### TABLE 12.1
Relationship between plasma and saliva concentrations of chlorthalidone in three humans

| Subject | Saliva | | | | |
|---------|--------|--------|--------|--------|
|         | Volume (ml) | pH | Sampling time (hr) | S/P ratio | |
| L.De    | 8.7    | 6.75  | 1.25   | 0.062 |
|         | 4.3    | 6.60  | 2.1    | 0.13 |
|         | 3.7    | 7.10  | 3.2    | 0.13 |
|         | 2.5    | 6.95  | 8.0    | 0.30 |
|         | 4.2    | 6.40  | 25     | 0.45 |
|         | 3.9    | 6.55  | 32     | 0.53 |
|         | 3.4    | 6.63  | 49     | 0.43 |
|         | 3.0    | 6.35  | 72.5   | 0.36 |
|         | 4.5    | 6.48  | 104    | 0.50 |
| Mean a  | 4.2    | 6.65  |        | 0.35 |
| ± SD    | ± 1.8  | ± 0.25|        | ± 0.16|
| T.Ho    | 2.2    | 7.25  | 1.0    | 0.029 |
|         | 3.2    | 7.15  | 2.3    | 0.14 |
|         | 2.4    | 7.42  | 2.65   | 0.14 |
|         | 2.3    | 7.22  | 4.0    | 0.12 |
|         | 3.6    | 6.90  | 8.0    | 0.21 |
|         | 2.8    | 7.36  | 12.0   | 0.23 |
|         | 3.7    | 7.12  | 25.3   | 0.50 |
|         | 2.9    | 7.18  | 49     | 0.25 |
|         | 2.2    | 7.35  | 73     | 0.27 |
|         | 2.9    | 7.12  | 97     | 0.20 |
| Mean a  | 2.8    | 7.21  |        | 0.23 |
| ± SD    | ± 0.6  | ± 0.15|        | ± 0.12|
| M.O     | 3.9    | 6.60  | 0.83   | 0.043 |
|         | 3.2    | 6.70  | 1.9    | 0.13 |
|         | 2.5    | 6.77  | 2.9    | 0.12 |
|         | 5.3    | 6.75  | 6.8    | 0.13 |
|         | 5.0    | 7.08  | 12.0   | 0.18 |
|         | 4.2    | 6.82  | 24.8   | 0.10 |
|         | 4.5    | 6.77  | 48.5   | 0.097|
|         | 4.2    | 6.70  | 96.5   | 0.089|
| Mean a  | 4.1    | 6.77  |        | 0.12 |
| ± SD    | ± 0.9  | ± 0.14|        | ± 0.03|

*a* saliva samples obtained at ca. 1 hr were not included in the calculation of the mean saliva over plasma concentration ratios
μg/ml range (Dieterle et al., 1976), so that the concentration of the drug in plasma water can be safely assumed to be approximately one-quarter of the total plasma concentration shown in Fig. 12.1.

Other factors obviously caused the large inter- and intraindividual differences in the S/P ratio of chlorthalidone. By analogy with other excretory organs active transport processes might be involved also in the salivary glands, as they are known to operate for sulfonamide diuretics in kidney and liver (Beyer and Baer, 1961; Schanker, 1968). In this case endogenous substrates would compete with the drug for transport and thereby could cause an irregular excretion rate. On the other hand, it is conceivable that the molecular volume of chlorthalidone (MW 338.8) did not facilitate rapid transport across the glandular membrane, which could be in agreement with a relatively slow transport rate through the red blood cell membrane (Chapter 8). Also the high abundance of carbonic anhydrase in parotid salivary glands (Maren, 1967) could have been of influence because chlorthalidone strongly binds to carbonic anhydrase in vitro (Dieterle et al., 1976; Chapter 8). Acetazolamide, however, the only other sulfonamide diuretic of which literature data on saliva levels were available (Wallace et al., 1977), showed a much more consistent correlation between its plasma and saliva concentrations, which could be due to its smaller molecular weight (222.2).

In conclusion, it has to be stated that the mechanism of salivary excretion of chlorthalidone, like that of several other drugs (see the review by Danhof and Breimer, 1978) is badly understood. Regardless this mechanism, however, a consistent relationship between saliva and plasma concentrations of chlorthalidone does apparently not exist (although a change in this situation after multiple dosing cannot be excluded). We have to conclude, therefore, that saliva measurements constitute no useful means for obtaining reliable pharmacokinetic parameters of chlorthalidone in single dose studies with this drug.

SUMMARY

Saliva and plasma concentrations of chlorthalidone were determined in three healthy human subjects during 100 hours following single intravenous doses of 50-60 mg. There was a large inter- and intraindividual variation in the saliva over plasma concentration ratio. The subject means were: 0.35 ± 0.16 S.D. (range 0.13-0.53), 0.23 ± 0.12 S.D. (range 0.12-0.50), and 0.12 ± 0.03 S.D. (range 0.09-0.18). It was concluded that saliva measurements are not suitable for pharmacokinetic single dose studies with chlorthalidone.
REFERENCES


CHAPTER 13

PHARMACOKINETICS OF CHLORTHALIDONE
DURING CHRONIC ADMINISTRATION

INTRODUCTION

Diuretic drugs need to be administered in general over long periods of time, especially if used in antihypertensive therapy. Chlorthalidone may be regarded as one of the most widely prescribed drugs in this field, certainly in The Netherlands, where its share in treatment of mild to moderate hypertension was estimated at about 70% of the total number of prescriptions of drugs*. A detailed study of the accumulation kinetics of chlorthalidone in man was, therefore, considered to be of major interest.

When linear pharmacokinetic processes prevail, accumulation of drug concentration until steady-state, can be predicted from the pharmacokinetic constants obtained in single dose studies (van Rossum and Tomey, 1970, van Rossum, 1971). Such situation implies that no alterations in plasma clearance and volumes of distribution occur, when single dose kinetics are extrapolated to higher concentration. Some exceptions to linear kinetics have been reported until now. The clearance of the analgesic salicylic acid in man proceeds via saturable metabolic pathways, giving rise to concentration-dependent elimination rate in the therapeutic dose range (Levy et al., 1972; van Ginneken et al., 1974). Analogous phenomena have been reported for ethanol (e.g. Wagner and Patel, 1972), ethylbiscoumacetate (van Dam, 1968), γ-hydroxybutyric acid (van der Pol et al., 1974) and phenytoin (e.g. Arnold and Gerber, 1970). More examples from literature were cited in publications by Wagner (1973) and by van Rossum and co-workers (1977). Another type of non-linearity can be found in saturable binding of drugs to plasma proteins or cellular constituents. Despite the fact that the theoretical possibility of occurrence of such phenomena had already early been recognized (Krüger-Thiemer, 1964; Wagner, 1973), only very few experimental examples have been provided up to now. Plasma concentrations of phenylbutazone (Burns et al., 1953) and warfarin (Kekki et al., 1977) did not increase linearly with increasing dose, but with less than the propor-

*Unpublished data over 1975 and 1976 obtained from a representative population of ca. 300,000 sick-fund insured subjects.
tional amount, indicating relatively reduced plasma protein binding at higher concentration. Capacity-limited binding to extravasal tissue has been proposed, tentatively, in case of a diazepam metabolite, N-desmethyldiazepam, to account for the much higher concentration of this metabolite attained in plasma after the second dose, as compared to the first one (Korttila et al., 1975).

These few cases may, however, suffice to indicate that non-linearities in either an upward or downward direction can show up from a multiple of causes. Therefore, for unequivocal explanation of non-linear pharmacokinetic behaviour always additional information should be available, which can be derived from, e.g. isolated liver perfusion, autoradiographic study, or in vitro protein binding. In vitro uptake of chlorthalidone by erythrocytes has been analysed in Chapter 8. Our findings pointed to occurrence of binding-sites with limited capacity, in the concentration range reached after therapeutic doses. Binding to intact red blood cells was approximately in agreement with the affinity constants and numbers of binding sites per molecule of two isoenzymes of carbonic anhydrase, as reported by Dieterle et al. (1976). The non-linear pharmacokinetic model of chlorthalidone, described in Chapter 7 (Fleuren and van Rossum, 1977), included this saturable binding in order to explain the time course of plasma and red blood cell concentrations after single doses in man. A decisive test for the validity of any hypothetical model is difficult to obtain, but a useful approach may be found in looking at what the model predicts. The model indicated that, after repetitive administration of chlorthalidone, red blood cell concentration would rise considerably less than expected from linear kinetic analysis of concentration-time curves, found after single doses. A comparison between steady-state levels, according to a linear and a non-linear accumulation process, is shown in Fig. 13.1.

For this purpose, hypothetical red blood cell concentration vs time data were generated by means of the non-linear chlorthalidone model, described in Chapter 7 by the differential equations of Appendix 7.2 and by the schematic drawing of Fig. 7.5. (Numerical values of the model parameters used here are given in Appendix 13.2 at the end of this chapter). The concentrations of the single dose curve of this hypothetical data set were subsequently regarded as if they would have been obtained in a real experiment. In that case, we could analyse these data according to linear pharmacokinetic equations. With the model parameters calculated we could simulate accumulation again, but now of course according to first-order processes only. This was done, and resulted in a higher plateau, as indicated in Fig. 13.1. It should be noticed that the difference between the two curves in the single dose phase is so small, that it would not be distinguished experimentally. This is in agreement with our observation, that separate concentration-time curves after single doses of chlor-
chlorthalidone concentration in red blood cells (µg/ml, log scale)

- expected plateau concentration, linear distribution mean $C_{ss, min} \times 31.5 \, µg/ml$
- non-linear distribution mean $C_{ss, min} \times 22.6 \, µg/ml$
- capacity-limited binding to red blood cells

Figure 13.1
Accumulation of red blood cell concentrations of chlorthalidone during multiple dosing according to two different pharmacokinetic models. The upper plateau (100%) was reached by superposition of single dose curves, when purely first-order processes were assumed. The lower plateau concentration (71% for the example chosen) was found when capacity-limited binding of chlorthalidone to erythrocytes was taken into account. For further explanation see text.

Chlorthalidone, can be described very well by linear pharmacokinetic equations, as outlined in Chapter 9. Notwithstanding this, it can be seen that a significant difference arises between the two approaches during repetitive administration.

The present study was undertaken to verify non-linear binding of chlorthalidone to red blood cells in the chronic situation. At first, the kinetic profile after administration of single doses had to be determined in order to obtain basic pharmacokinetic parameters, necessary for predicting steady-state concentration. The study was performed at two dosage levels in the same human subject in order to assure a wide concentration range.
**Drug administration**
A young, well-motivated male human subject (J. Se, 28 yr, 77 kg) with mild essential hypertension (pretreatment blood pressure, systolic/diastolic: 140/100 mm Hg, both in supine and standing position; average of two visits to the clinician) and no further disease, took part in the study after informed consent. The first part of the study consisted of a 50 mg dose schedule, in the second part a 100 mg dose regimen was used. The two trials were separated by an interval of 1 month. Details of the protocol are shown in Fig. 13.2. Commercial chlorthalidone tablets of 100 and 50 mg were administered (Hygroton®, Ciba-Geigy, Basle, Switzerland). On the first day of each trial, the tablet was taken together with about 200 ml of tap water at 9.00 A.M., after overnight fasting. No food or fluid, except water, was taken until 3-4 h after the dose. During chronic administration, the tablets were ingested once a day, usually at 9.00 A.M., and, although abstinence from food during several hours was, of course, not imperatively prescribed, subject J. Se was used to consume no breakfast in the morning. No other medication was used during both trials and during at least 1 month preceding them.

![Protocol of chronic pharmacokinetic study with chlorthalidone.](image)

---

**Sampling and analytical measurement**
In the single dose studies, venous blood samples of ca. 7 ml were taken, as described in Chapter 3, at the following approximate times (exact times were noted): 0, 1, 2, 3, 5, 7 and 24 hours and at 2, 3, 4, 7, 8 and 9 days after administration. During the period of repetitive doses, blood samples were
obtained mostly at 9.00 A.M., prior to intake of the next dose. In addition, on some days, which are indicated in Fig. 13.2, more than one sample was taken, viz. at noon (12.00 h) and at 5.00 P.M. (17.00 h). Urine was collected completely, in 24 h portions, during 10 days in the steady-state of each dose regimen, as depicted in Fig. 13.2.

The concentration of chlorthalidone in plasma, red blood cells and urine was determined as described in Chapter 3, with the exception that plasma was extracted at pH 9 instead of at pH 7.4, because the gas chromatograms from subject J. Se showed, occasionally, small interfering peaks of unknown origin, which were avoided by extracting the drug at higher pH than that usually employed (cf. Chapter 3). Creatinine in plasma and urine was determined with a modified Jaffé reaction, by using continuous flow analysis with dialysis and reaction with alkaline picrate (see, e.g. Henry et al., 1974).

**Pharmacokinetic analysis**

Plasma concentrations of chlorthalidone after administration of single doses were fitted to the exponential equation, according to a linear two compartment open model after oral administration, described in Chapter 1. Red blood cell concentrations were fitted according to a one compartment open model, with first-order absorption, see also Chapter 1. Plasma clearance and volumes of distribution ($V_1$ and $V_{dss}$) were calculated as described in Chapter 1. Under the assumption of linear, i.e. first-order processes, plasma concentration during repetitive doses, with constant dosage interval $Δt$, can be described by the following general equation (van Rossum and Tomey, 1970):

$$ C = \sum_{i=1}^{n} A_i \cdot \frac{-jΔt/τ_i}{1-e^{-Δt/τ_i}} \cdot e^{-t/τ_i} $$

(Eq. 13.1)

where $C$ represents plasma concentration at any time after the start of therapy, $n$ is the number of compartments of the body plus gastrointestinal tract, $A_i$ is the ith coefficient, $τ_i$ the ith time constant (reciprocal rate constant), and $j$ the number of doses that have been administered, while $t$ is the time after administration of the $j$th dose. When the dosage interval is not exactly constant, simulations can more easily be performed by numerical integration by means of a suitable computer program. Details of the procedure are described in Appendix 13.1 at the end of this chapter. A similar approach was used for generation of the curves shown in Fig. 13.1.
RESULTS

Single dose kinetics

The time course of plasma concentrations of chlorthalidone was biphasic, and could accordingly be described by means of a linear two compartment model with first-order absorption, in agreement with the results obtained from other human subjects, which have been dealt with in the Chapters 7, 9 and 10. Peak concentrations of chlorthalidone in red blood cells were reached at about 10 h after the dose in both single dose trials. At that time, concentrations were ca. 100 times higher than those in plasma. Similarly as described in Chapter 9, the red blood cell concentrations were fitted according to a linear open one compartment model with first-order absorption.

Some important pharmacokinetic parameters for subject J. Se, obtained from this linear analysis of plasma and red blood cell concentrations after both dose levels, are shown in Table 13.1.

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Plasma concentration</th>
<th>Erythrocyte concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5.10</td>
<td>438</td>
</tr>
<tr>
<td>100</td>
<td>11.20</td>
<td>935</td>
</tr>
</tbody>
</table>

Multiple dose kinetics

The height of the plateau level, predicted by using the first-order kinetic parameters from the single dose experiments, was not reached. Figs. 13.3 and 13.4 compare the simulated accumulation with actual measurement. During the 50 mg dosage regimen, minimum steady-state concentration was 86% and 57% of the expected levels in plasma and erythrocytes, respectively. Following 100 mg doses, the difference was even more pronounced, the minimum plasma and red blood cell concentrations during steady-state being 59% and 50% of the predicted values, respectively.
Figure 13.3
Plasma and red blood cell concentrations of chlorthalidone, on semi-logarithmic scale, after both single and multiple 50 mg oral doses. The concentrations measured after administration of the single dose were fitted to linear pharmacokinetic model equations and the parameters obtained were used to simulate concentration-time curves during steady-state and thereafter. Whereas a reasonable agreement was found, at this dosage level, between predicted and observed plasma concentrations, much lower chlorthalidone concentrations than predicted were found in the red blood cells, indicating reduced uptake of this drug in the erythrocytes at higher concentration. See also text at Fig. 13.4.
Figure 13.4

Comparison of observed and predicted steady state concentration of chlorthalidone during daily doses of 100 mg in man. The continuous lines represent concentration-time curves, predicted on basis of curve-fitting of the single dose data according to first-order kinetics. The broken lines connect plateau concentrations measured just before intake of the next dose and correspond therefore to minimum steady-state levels. Judged from the total profile of plasma concentrations during steady-state (including a few peak concentrations, see lower panel), a somewhat smaller difference between predicted and observed values might become apparent, if the average steady-state plasma concentration could be calculated exactly. Accumulation of red blood cell concentration of chlorthalidone, however, was obviously not in agreement with linear binding to red blood cell constituents. Compare with text at Fig. 13.3.
*Urinary excretion during steady-state*

The 24-hours cumulative amounts of chlorthalidone in urine, during 10 days of both steady-state periods, are presented in Table 13.2. A mean total of 38.1% of dose was recovered during the 50 mg dosage regimen, and a mean of 47.1% of dose during the 100 mg dose schedule. This difference was statistically significant (p < 0.025, Wilcoxon’s signed ranks test).

The different urinary recoveries were not correlated with renal plasma clearance of creatinine: urinary amounts of creatinine were 17.2 (± 0.7 S.D.) mmol/24 hr (n = 10) and 17.0 (± 0.9 S.D.) mmol/24 hr (n = 10) during the 50 and 100 mg trial respectively. The mean plasma creatinine concentration was 72.3 (± 3.7 S.D.) μmol/l (n = 7) and 73.1 (± 3.5 S.D.) μmol/l (n = 7) during the 50 and 100 mg dosage regimen, respectively, so that a creatinine clearance resulted of 165 ± 7 ml/min (mean ± S.D., n = 7) and 159 ± 11 ml/min (mean ± S.D., n = 7) during repetitive administration of 50 and 100 mg chlorthalidone respectively. Also the in-

| TABLE 13.2 |

Urinary excretion of chlorthalidone during 10 consecutive days of steady-state\(^a\) under two different dosage regimens, in the same human subject.

<table>
<thead>
<tr>
<th>50 mg chlorthalidone daily(^b)</th>
<th>100 mg chlorthalidone daily(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine flow (ml/24 h)</td>
<td>Urine pH (average)</td>
</tr>
<tr>
<td>1360</td>
<td>6.38</td>
</tr>
<tr>
<td>1375</td>
<td>5.96</td>
</tr>
<tr>
<td>1065</td>
<td>6.35</td>
</tr>
<tr>
<td>1265</td>
<td>6.48</td>
</tr>
<tr>
<td>1090</td>
<td>6.33</td>
</tr>
<tr>
<td>1145</td>
<td>5.88</td>
</tr>
<tr>
<td>1240</td>
<td>6.16</td>
</tr>
<tr>
<td>1075</td>
<td>6.37</td>
</tr>
<tr>
<td>1250</td>
<td>6.22</td>
</tr>
<tr>
<td>1420</td>
<td>6.00</td>
</tr>
</tbody>
</table>

mean(± S.D.)

38.2(± 5.1) 

38.2(± 5.1) 

mean(± S.D.)

47.5(± 5.3) 

\(a\) = first collection on day no. 12 of multiple dosing

\(b\) = creatinine clearance during 50 mg/day: 165(± 7 S.D.) ml/min; during 100 mg/day: 159 (± 11 S.D.) ml/min. Difference not significant.
individual daily excreted amounts of creatinine and chlorthalidone showed no correlation.

Renal plasma clearance of chlorthalidone could be calculated only approximately, because most blood samples had been taken at the ends of the urine collection periods, so that the complete time course and, therefore, the exact areas under the plasma concentration curves during steady-state were not known. Upper estimates, obtained by using the average minimum plasma concentration, resulted in values of, at most, 6.63 l/h (111 ml/min) and 9.9 l/h (165 ml/min) for the 50 mg and 100 mg dose regimen, respectively.

Therapeutic effect
Regular blood pressure recordings indicated no significant decrease in blood pressure for the subject under study. Representative values at the end of both treatment periods were: 126/94 and 126/100 mm Hg after 50 mg repetitive doses, and 142/88 and 148/96 mm Hg following 100 mg dosage of chlorthalidone (recorded in supine and standing position respectively).

DISCUSSION

Blood pressure response
Despite considerable concentrations of chlorthalidone in blood, no or hardly any reduction in blood pressure was achieved. The young age of the subject (28 yr) may have been a factor here, as failure of young hypertensive patients to respond to diuretic treatment has been described (Gordon et al., 1977). In that report it was indicated that young people have a greater ability to compensate for the diuretic-induced loss in plasma volume by homeostatic mechanisms, e.g. mediated by a larger vascular flexibility as compared to that in older patients.

Absorption during chronic administration
If linear differential equations would adequately describe the pharmacokinetic behaviour of chlorthalidone, steady-state concentration would be determined solely by the dose (D), the dosage interval (Δt) and the plasma clearance (k_{Ce1}) (van Rossum, 1971):

$$
\bar{C}_{pL,ss} = \frac{F \cdot D}{k_{Ce1} \cdot \Delta t}
$$

(Eq.13.2)
The oral bioavailability, F, is in principle unknown, therefore, also its influence on the steady-state concentration attained. Therefore, prior to drawing conclusions on non-linearity of drug distribution, some remarks concerning drug absorption have to be made. As is described in Chapter 9, about 70-80% of the dose is absorbed from the gastro-intestinal tract after administration of single 50 mg doses of chlorthalidone. A similar percentage of absorption after single 100 mg doses was indicated by the findings of Chapter 10, showing that cumulative urinary excretion of chlorthalidone increased linearly with dose up to the 100 mg level. Although no urine was assayed in the single dose phase of the present study, the areas under the curves of plasma and erythrocyte concentration after the 50 and 100 mg dose were approximately in a ratio 1:2 (see Table 13.1). This confirms that large variation in the degree of absorption between the two different single doses in subject J. Se did not occur.

During chronic administration of chlorthalidone, a mean daily amount of 38.1% and 47.5% of dose was recovered, in the 50 mg and 100 mg schedule, respectively, see Table 13.2. These figures fell in the range of total urinary amounts encountered after single doses in normal human volunteers: 44.4 (± 6.8 S.D.)% of dose after 50 mg and 43.1 (± 4.7 S.D.)% of dose after 100 mg of chlorthalidone (Fleuren et al., 1979; Chapter 10). Suppose, however, that the lower steady-state concentration, being even ca. 50% of the expected value (Fig. 13.4), would still have been caused by an absorption degree, which was systematically reduced as compared to the single dose situation. In that case, the theoretical cumulative urinary amount, expected for that single dose experiment, would have been twice as high as the daily amounts found during steady-state, i.e. 76% (50 mg) and 94% (100 mg) of dose. Such high values have never been observed after oral administration of single doses, see Chapter 10, or the report by Riess et al. (1977). Concludingly, a different degree of absorption during chronic administration must be considered as a highly improbable cause for the reduced steady-state blood levels of chlorthalidone.

Red blood cell binding

Our observations can better be explained on basis of capacity-limited binding of chlorthalidone to red blood cells. Ever since its introduction, chlorthalidone was known as an in vitro inhibitor of red cell carbonic anhydrase, although the concentration at which 50% inhibition was observed, was about 10 times higher than that of acetazolamide (Maren, 1967; Pulver et al., 1962). In agreement with this, Beermann et al. (1975) demonstrated that chlorthalidone was attached to the carbonic anhydrase fraction of the red blood cell proteins; the drug could be displaced from its erythrocyte binding sites by acetazolamide, both in vivo and in vitro.

Carbonic anhydrase is a metallo-protein of molecular weight ca 30000,
containing one zinc atom per molecule, which occurs, so far as erythrocytes are concerned, exclusively in cell water. The enzyme has a wide-spread distribution over mammalian tissues, especially high concentrations being found, besides in red blood cells, in kidney cortex, stomach parietal cells, eye lens and retina, parotid salivary glands, and the choroid plexus of the brain. More information on its occurrence, physiological function and biochemical properties can be found in the extensive reviews by Maren (1967) and Carter (1972). Some points of interest for the present investigation have to be mentioned here. Two major isoenzymes of human red cell carbonic anhydrase, so-called HCA-B and HCA-C, have been isolated. Besides in physicochemical properties, these isoenzymes differ greatly in their catalytic efficiency. HCA-C possesses a carbon dioxide hydratase activity, which is 20-40 times that of HCA-B on a molar basis. The high activity isoenzym has on the other hand a much lower abundance than the low activity isoenzym. The intracellular concentration of HCA-C was reported as ca. 20 μM, versus ca. 140 μM for HCA-B, see also Wistrand and Baathe (1968). Furthermore, it was observed that HCA-C function was inhibited by smaller concentrations of acetazolamide and other sulfonamide inhibitors than those needed for inhibition of HCA-B. Correspondingly, higher association constants were measured for the binding of carbonic anhydrase inhibitors to the HCA-C isoenzyme (Maren, 1967).

The binding of chlorthalidone to the pure isoenzymes was determined by Dieterle et al. (1976). The association constant was 5.7x10⁶ l/mole for HCA-C and 2.4x10⁶ l/mole for HCA-B. The in vitro binding of chlorthalidone to intact red blood cells, described in Chapter 8 of this thesis, was in qualitative agreement with the above data. The amount of drug involved in the strongest binding might correspond with the known molar amount of the isoenzyme HCA-C. So, we proposed that this small HCA-C fraction would represent the binding sites of limited capacity in the non-linear pharmacokinetic model, described in Chapter 7 (Fleuren and van Rossum, 1977). The prediction made according to this model with respect to accumulation of chlorthalidone concentration during multiple dosing, appears to be fulfilled, at least qualitatively, by the experimental data shown in Figs. 13.3 and 13.4.

In addition, it can be observed from Fig. 13.4 that not only the steady state concentration in erythrocytes but also that in plasma was smaller, during the 100 mg dose regimen, than expected if no other factors than the distribution of the drug between plasma and red cells were in play. An obvious co-determinant of the concentration in plasma is the renal plasma clearance. It should be noticed in this context that the renal plasma clearance of chlorthalidone during repetitive administration of the 100 mg
doses was larger than in the 50 mg dosage period. This aspect is discussed separately in the following section.

Renal clearance

We can offer no definite explanation at present for the elevated renal clearance of chlorthalidone during the higher - 100 mg - dose regimen as compared to the lower - 50 mg - one. This effect was possibly secondary to the combination of higher urine pH and flow, observed in the latter study (Table 13.2). In turn, this average 24-hours urine pH of 6.7 could have been caused by a certain degree of renal carbonic anhydrase inhibition at higher doses (see below). On the other hand, the maximum capacity of distal kidney tubular cells to reabsorb the drug might be approximated during this dose schedule. In single dose studies with chlorthalidone, described in Chapter 10, a decrease in the percentual urinary excretion and also, in so far as a comparison could be made, in renal clearance occurred with increasing dose. Unfortunately, we have no data at hand to compare the single with the multiple dose situation in the present study. It is, however, conceivable that the physiological status of the kidney during chronic medication is very much different from that in the beginning, e.g. with regard to homeostatic mechanisms, so that extrapolation from the single dose situation, as proposed in the discussion section of Chapter 10, would be complicated by additional factors. More experimental work with chlorthalidone and other thiazides is needed with respect to the renal handling of diuretics during chronic administration.

Therapeutic meaning

Maren (1963) and also Wistrand and Baathe (1968) have pointed out that a very large excess of carbonic anhydrase activity in relation to the physiological need, at least 100-fold, is apparent in many tissues, including red cells and kidney. Therefore, a fractional inhibition of more than 99.5% or, equivalently, an almost complete receptor occupancy, would be required to achieve only a 50% inhibition of the in vivo enzyme function. This would explain why severely toxic effects of carbonic anhydrase inhibitors are not encountered normally in diuretic therapy. Undesirable side-effects, however, attributed to carbonic anhydrase inhibition, do frequently occur during long term treatment with sulfonamide diuretics. Although it is a general opinion nowadays, that renal carbonic anhydrase does not play the first role in the biochemical mechanism of action of diuretic agents (Meng and Loew, 1974; Peters and Roch-Ramel, 1969; see also Chapter 1), inhibitory effects appear following higher doses of these drugs. Tweeddale et al. (1977), for instance, compared the antihypertensive and biochemical effects of different daily doses of chlorthalidone, ranging from 25-200 mg. While maximum blood pressure reduction was achieved already with 25 or
50 mg doses in two third of the patients, side-effects, e.g. the decrease in serum potassium concentration, strongly increased with dose. Minimal biochemical changes were observed with daily doses of 50 mg or less.

To account for these findings, we would like to suggest the following. The strong binding sites of chlorthalidone in red blood cells, tentatively associated with one of the isoenzymes of carbonic anhydrase, HCA-C, as described in Chapter 8, act as a depot and keep free concentrations low. When their capacity is exceeded, e.g. above daily doses of 50 mg, disproportionally higher amounts of drug become available to the kidneys. It is tempting to speculate in this context that the height of the red blood cell concentration of chlorthalidone operates merely as an indicator for the probability, that side-effects of this drug are going to occur. Further monitoring of red blood cell concentrations in relation to diuretic effects will be required to support this view.

SUMMARY

A young, mildly hypertensive subject received single and multiple oral doses of chlorthalidone, according to both a 50 and a 100 mg dosage regimen. Each trial took 45 days, which were separated by a 1 month interval. At first, plasma and red blood cell concentrations were followed during 10 days after the single dose, in order to obtain pharmacokinetic model parameters for prediction of steady-state levels. Thereafter, the drug was administered once a day during three weeks. In this period urinary excretion of chlorthalidone was also determined. After cessation of repetitive dosing, the decline of plasma and red blood cell concentrations was measured over 14 days. The average 24-hours urinary excretion of unchanged chlorthalidone, at steady state, was 38.2 (± 5.1 S.D.)% of dose, and 47.5 (± 5.3 S.D.)% of dose, during the 50 and 100 mg dosage regimens respectively. This difference was statistically significant, but not correlated with creatinine clearance.

Steady-state concentrations of chlorthalidone in plasma, and especially those in red blood cells, were lower than the values predicted by use of a linear pharmacokinetic model with parameters obtained from the single dose studies. The difference between the expected and observed values became more pronounced at the 100 mg dose level. Thus, during the 50 mg dosage regimen, a minimum steady-state plasma concentration of, on an average, 0.12 µg/ml was observed, while 0.14 µg/ml had been predicted. For erythrocytes a value of 8.9 µg/ml was found, instead of the predicted 15.5 µg/ml. During the 100 mg dosage regimen, a minimum steady-state concentration of 0.34 µg/ml in plasma had been predicted, while only 0.20
μg/ml was found. In erythrocytes a value of 34.3 μg/ml had been predicted, but only 17.1 μg/ml, on an average, was observed.

It was concluded that capacity-limited binding of chlorthalidone to red blood cells, probably to an isoenzyme of carbonic anhydrase, was responsible for this non-linear increase of chlorthalidone concentration during chronic administration.

APPENDIX 13.1

The following first-order differential equations describe the time course of plasma and red blood cell concentrations, according to a open two- and a one compartment model, respectively.

**Plasma concentration**

\[ \frac{dQ_o}{dt} = -r_a \cdot Q_o \]

\[ \frac{dQ_1}{dt} = r_a \cdot Q_o - (r_{el} + r_{12}) \cdot Q_1 + r_{21} \cdot Q_2 \]

\[ \frac{dQ_2}{dt} = r_{12} \cdot Q_1 - r_{21} \cdot Q_2 \]

**Red blood cell concentration**

\[ \frac{dQ_o}{dt} = -r_a \cdot Q_o \]

\[ \frac{dQ_1}{dt} = r_a \cdot Q_o - r_{1el} \cdot Q_1 \]

In these equations \( Q_0, Q_1 \) and \( Q_2 \) denote the quantities of drug in the absorption compartment, central compartment (\( V_1 \)), and peripheral compartment, resp.; \( r_a, r_{12}, r_{21} \) and \( r_{1el} \) are first-order rate constants of absorption, transfer from central to peripheral compartment and vice versa, and elimination from the central compartment, respectively.
During the 50 mg dose schedule, plasma concentration was simulated with: 
\[ r_a = 12.99, r_{el} = 2.51, r_{12} = 8.51, r_{21} = 2.21 \text{ (all in days}^{-1}) \], and \( V_1 = 97.7 \text{ l.} \) Red blood cell concentration with: 
\[ r_a = 7.10, r_{el} = 0.361 \text{ (both in days}^{-1}) \], and \( V_1 = 7.73 \text{ l.} \)

During the 100 mg dose schedule, plasma concentration was simulated with: 
\[ r_a = 15.85, r_{el} = 1.284, r_{12} = 7.23, r_{21} = 4.66 \text{ (all in days}^{-1}) \] and \( V_1 = 174.9 \text{ l.} \) Red blood cell concentration according: 
\[ r_a = 4.70, r_{el} = 0.391 \text{ (both in days}^{-1}) \] and \( V_1 = 7.73 \text{ l.} \)

In addition, the following functions were used:
\[ Q_0(0) = \text{dose}; C_1 = Q_1/V_1 \]
\[ Q_0(t+h) = Q_0(t) + I \]
\[ I = \text{dose for } t<T_j<t+h; h = 0.005 \text{ day (step width)} \]
\[ I = 0 \text{ for } t<T_j<t+h \]
\[ T_j = \Sigma \Delta t_i \text{ (from } i=1,...,j), \text{ where } j = 1, ..., 21; T_{21} = 30 \text{ days} \]
\[ \Delta t_1 = 10 \text{ days}; t_j \sim 1 \text{ day for } i \neq 1, \Delta t_i = \text{dosage interval} \]

**APPENDIX 13.2**

The plot of erythrocyte concentrations of chlorthalidone vs time, shown in Fig. 13.1, according to the non-linear model of Chapter 7, was simulated with the following model constants: \( D = 100 \text{ mg}, V_0 = 51, V_1 = 175 \text{ l, } V_2 = 1.3 \text{ l, } V_3 = 2.71, k_{c12} = 40 \text{ l/hr, } k_{c21} = 0.5 \text{ l/hr, } k_{c13} = 20 \text{ l/hr, } k_{c31} = 0.5 \text{ l/hr, } k_{ca} = 10 \text{ l/hr, } k_{cel} = 7.8 \text{ l/hr, } A = 0.15 \text{ (mg/l)}^{-1, B = 6 mg/l.} \) The meaning of these parameters can be found at the original description of the model in Chapter 7.

For generation of the levels during steady-state, a procedure analogously to that described in Appendix 13.1 was employed, but now of course by use of the differential equations for the non-linear model, as described in Appendix 7.2 in Chapter 7.

**REFERENCES**


Korttila, K., Mattila, M.J., Linnoila, M.: Saturation of tissues with N-demethyl-diazepam as cause for elevated serum levels of this metabolite after repeated administration of diazepam. *Acta pharmacol. toxicol.* 36, 190-192 (1975)


INTRODUCTION

Since its introduction in 1967 (Horstmann et al., 1967) the sulfonamide diuretic mefruside (Baycaron®) has been established as a diuretic with an intermediate duration of action (Brogden et al., 1974; Schwab and Im- mich, 1967; Wilson and Kirkendall, 1970). The pharmacokinetic background of these clinical observations was, however, until now unclear. Although the distribution and metabolism of mefruside in rats and dogs have been well-described (Duhm et al., 1967; Schlossmann and Pütter, 1973), kinetic data of this drug in man remained very scarce. In one human study (Schlossmann, 1967) the use of a spectrophotometric assay allowed measurement only of the sum of mefruside plus metabolites. In another report (Duhm et al., 1967), C\textsuperscript{14}-mefruside was administered to a single subject, and, after fractionation of urinary radioactivity, a parallel was drawn, tentatively, with the metabolic pattern in rats. In short, these animal studies had shown that oxidation of the tetrahydrofuran ring of mefruside accounted for the largest part of metabolites (Duhm et al., 1967). The main product, 5-oxo-mefruside, alternatively called mefruside-lactone, existed in equilibrium with its open acid analogue. See Fig. 14.1. Interconversion of the two metabolite forms was strongly catalyzed in vivo, so that the open acid form constituted an important secondary metabolite (Pütter and Schlossmann, 1972; Schlossmann and Pütter, 1973).

In the Chapters 4 and 5 gas chromatographic methods were described for the specific determination of mefruside and both oxidized products in human plasma, red blood cells and urine, and these two compounds were identified as metabolites of mefruside in man (Fleuren et al., 1979a, 1979b). Simultaneous analysis of mefruside and metabolites seemed to be relevant, because the lactone and the open acid compound have been reported to give the same diuretic effect as the parent drug (Meng and Kroneberg, 1967). Moreover, preliminary experiments had indicated that both metabolites were excreted into human urine (Chapter 5; Fleuren et al., 1979b), suggesting that they might be very important for diuretic action.
Figure 14.1
Structural formulae of mefruside, 5-oxo-mefruside and its open acid analogue.

MATERIALS AND METHODS

Drug administration and sampling of blood and urine.

Eight young male volunteers took part in the study. They were aged 21-24 yr, and weighed 61-84.5 kg (detailed body weight data included in Table 14.1). All were healthy and took no other medication for at least one month before or during the study. Each subject received a single oral dose of 25 or 50 mg of mefruside as commercial tablets (Baycaron®, 25 mg, Bayer) together with about 250 ml of tap water at 9.00 A.M., after an overnight fast. A 25 mg dose was taken by two, and a 50 mg dose by six subjects, as indicated in Table 14.1.

Blood samples of ca. 7 ml were drawn by venepuncture from an antecubital vein, usually at the following approximate times after the dose (exact times were noted): 0, 0.5, 1, 1.5, 2, 3.5, 5.5, 8, 14, 24, 32, 48 and 56 h. All blood samples were heparinized and centrifuged immediately after collection, followed by rapid separation of plasma from red blood cells, as described in the Chapters 4 and 5. Both blood fractions were kept frozen at -20°C until assay. Urine was collected completely in separate portions,
usually for 56 h. Times of voiding were noted, urine volumes and pH were measured, and aliquots of each portion were frozen until determination.

**Assay of mefruside and metabolites**

Concentrations of mefruside, mefruside-lactone and its open acid analogue were determined separately in plasma, red blood cells and urine by gas chromatography with nitrogen detection (Chapters 4 and 5). From plasma samples, expected to contain a very low mefruside concentration, i.e. ca. 2.5-7.5 ng/ml, 3 ml portions of plasma were used for assay. The distribution of mefruside and metabolites between plasma and red blood cells is temperature-dependent, so that irregular concentration-time curves can be found, if no precautions in the analytical procedure are taken (Fleuren et al., 1979a, 1979b). By employing a 3 min centrifugation at 20°C starting immediately after collection of the blood samples, the plasma concentration of mefruside was found to be constantly ca. 15% lower than that present at 37°C. Therefore, estimates of half-lives were not influenced by this procedure (see Chapter 4). In the present study, the uncorrected plasma concentrations were used for pharmacokinetic calculations.

Glucuronidase treatment of urine was carried out by incubating 1 ml portions of urine with 2000 Fishman units of β-D-glucuronidase containing arylsulfatase activity from *Helix pomatia* (Sigma, St. Louis, Mo., USA) during 16 hours at 37°C. Before incubation, urine samples had been adjusted to pH 5.2 with a few drops of dilute aqueous HCl and buffered with 1 ml of 0.1 M Walpole acetate buffer (pH 5.2).

**Pharmacokinetic analysis**

Plasma concentration vs. time data of mefruside were fitted according to exponential equations, corresponding to linear open compartment models with first-order absorption, similarly as described in Chapter 9 for pharmacokinetic studies with chlorthalidone (Fleuren et al., 1979c). Because preliminary graphical estimation indicated that the data could be described by either a one or a two compartment model, decision in favour of the most appropriate model equation for each subject was made by the f-test for goodness of fit (see e.g. Boxenbaum, 1974) which resulted in choice of a two compartment model for five subjects and a one compartment model for three, as reported in the Results section.

For curve-fitting the computer program FARMFIT* was used, which yielded the exponential terms, i.e. $K_a$, $\alpha$, $\beta$, and the coefficients $A$ and $B$ of the model equations. This program provided also a measure of the relative

* FARMFIT, a non-linear curve-fitting program in use at the Computer Centre of the University of Nijmegen. Details are given in Chapter 1.
error in the parameter estimates, by dividing the asymptotic standard deviation of the estimated parameter by the computer-estimated parameter itself (times 100 gives relative error in %). A relative error of 4% was taken into account for each concentration value \( C_i \): \( \text{weight}_i = 1/((0.04 \ C_i)^2) \), because this was the mean standard deviation of the gas chromatographic assay. Terminal half-lives were calculated as \( t_{1/2} = 0.693/\beta \), where \( \beta \) represents the smallest rate constant (reciprocal time constant) for each fitted curve. The plasma clearance (\( k_{\text{ce}} \)), the volume of the central compartment (\( V_1 \)) and the volume of distribution at steady state (\( V_{\text{dss}} \)) were obtained directly from the coefficients and exponentials of the fitted model equations, similarly as described in Chapter 9. It should be noticed that these parameters can be calculated from oral administration data only as \( V_1/F \), \( V_{\text{dss}}/F \) and \( k_{\text{ce}}/F \), as indicated in Table 14.1, because the bioavailability \( F \), i.e. the fraction of dose coming into the general circulation, is not actually known.

In one subject, F. So., irregular absorption of mefruside did not allow use of a curve-fitting procedure for obtaining pharmacokinetic parameters, as will be shown in Figure 14.5 in the Results section. Instead, the terminal half-life was found by non-linear regression analysis of the final straight part of the curve, and the plasma clearance and the total volume of distribution (divided by \( F \)) were calculated as \( D/\text{AUC} \) and \( D/\beta \cdot \text{AUC} \), respectively, where \( D \) represents the dose and \( \text{AUC} \) the area under the plasma concentration vs. time curve, extrapolated to infinity from the last data point. The \( \text{AUC} \) had been obtained by use of the trapezoidal rule.

The renal plasma clearance of mefruside was measured by dividing the amount of drug excreted in urine by the corresponding area under the plasma concentration curve during the period that the plasma concentration could be accurately analysed, i.e. down to ca. 5 ng/ml using 2 ml of plasma (Fleuren et al., 1979a; Chapter 4).

The apparent terminal half-lives of 5-oxo-mefruside and its open acid analogue were calculated by non-linear regression analysis of the final part of the concentration-time curves and urinary excretion rate vs. time plots. The plots of urinary excretion rate vs. time were extrapolated to obtain the total urinary recovery of mefruside and metabolites at infinite time.

RESULTS

Pharmacokinetics of mefruside in plasma

Mefruside was rapidly absorbed with peak plasma concentrations of 68-128 ng/ml reached at 1.5-5.5 h (mean 2.6 h) after oral doses of 50 mg in six subjects. In the two subjects, who had received a 25 mg dose, peak con-
centrations of 76 and 84 ng/ml occurred at 2 and 2.5 h, respectively. After the top the concentration-time curves were biphasic in five subjects. In the three other subjects terminal elimination was so fast with respect to distribution, that initial decay, viz. the first phase of disappearance visible in the afore-mentioned five subjects, could not be distinguished in them as a separate phase. To illustrate this difference between subjects, typical plasma concentration vs. time curves on semi-logarithmic scale are shown in Fig. 14.2. Considerable inter-individual variation in $t_{1/2}$, over a 4-fold range, was found. The pharmacokinetic parameters, obtained by fitting the data according to either a one or a two compartment open model, are given in Table 14.1. It can be seen from this table that the variation in terminal half-life, from 2.9-12.5 h, is reflected mostly in differences in the plasma clearance ($k_{Cel}$), which ranges from 22.5-1291/h, whereas intersubject differences in the total volume of distribution ($V_{dss}$) are much less pronounced, ranging from 314-518 liters.

![Figure 14.2](image)

Computer-fitted plasma concentration curves of mefruside, on semi-logarithmic scale, after oral administration of single 50 mg doses to three human subjects. A ca. 4-fold intersubject difference in elimination half-lives was observed.
**TABLE 14.1**

Open pharmacokinetic model parameters describing plasma concentration of mefruside after oral administration to eight healthy human subjects (Rel. errors in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>One compartment</th>
<th>Two compartments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects</td>
<td>Subjects</td>
</tr>
<tr>
<td></td>
<td>J.Cl</td>
<td>J.Da</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Dose/weight (mg/kg)</td>
<td>0.78</td>
<td>0.68</td>
</tr>
<tr>
<td>Lag time (h)</td>
<td>0.38</td>
<td>0.01</td>
</tr>
<tr>
<td>$K_a$ (h$^{-1}$)</td>
<td>2.97</td>
<td>1.01</td>
</tr>
<tr>
<td>($38%$)</td>
<td>($9.9%$)</td>
<td>($12%$)</td>
</tr>
<tr>
<td>$\alpha$ (h$^{-1}$)</td>
<td></td>
<td>($63%$)</td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.204</td>
<td>0.158</td>
</tr>
<tr>
<td>($6.4%$)</td>
<td>($3.2%$)</td>
<td>($2.3%$)</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>3.4</td>
<td>4.4</td>
</tr>
<tr>
<td>$A$ (mg/l)</td>
<td>0.521</td>
<td>0.210</td>
</tr>
<tr>
<td>($294%$)</td>
<td>($528%$)</td>
<td>($183%$)</td>
</tr>
<tr>
<td>$B$ (mg/l)</td>
<td>0.171</td>
<td>0.138</td>
</tr>
<tr>
<td>($10.4%$)</td>
<td>($6.5%$)</td>
<td>($5.7%$)</td>
</tr>
<tr>
<td>$V_1/F$ (l)</td>
<td>210</td>
<td>243</td>
</tr>
<tr>
<td>$V_{dss}/F$ (l)</td>
<td>314</td>
<td>428</td>
</tr>
<tr>
<td>$k_{Cel}/F$ (1/h)</td>
<td>64.1</td>
<td>67.4</td>
</tr>
</tbody>
</table>

$^a$ $V_d\beta$ calculated instead of $V_{dss}$ for subject F.So, see text for further explanation
Uptake of mefruside in red blood cells

Mefruside concentration in erythrocytes was 20 to 40 times higher than corresponding plasma concentration. Peak concentrations, 1.83-3.53 µg/ml after 50 mg doses, and 1.49 and 2.50 µg/ml after 25 mg of mefruside, were reached at the same times as those at which peak concentrations in plasma had been found. The time-averaged ratio of red blood cell concentration over plasma concentration was closely equal to 30 in five subjects. Mean ratios (± S.D.) were: 29.4 (± 3.0) in subject H.Ba, 29.9 (± 6.7) in subject J.Cl, 29.7 (± 3.5) in subject A.Ro, 28.9 (± 2.5) in subject S.Si, and 29.9 (± 5.1) in subject F.So. In the subjects J.Da, K.Ko and P.Pe, the mean ratios were 35.5 (± 3.7), 36.2 (± 3.9) and 20.1 (± 2.6), respectively.

At and around the top of the concentration time curves, the red blood cell over plasma concentration ratio was systematically ca. 10-15% lower than the time-averaged value in each subject. The ratio became constant, however, after this time, so that a parallel terminal decay of the concentration in both blood fractions resulted. Figure 14.3 shows red blood cell con-

![Figure 14.3](image)

*Figure 14.3*

Red blood cell concentrations of mefruside are ca. 30 times higher than corresponding plasma concentrations and provide virtually the same information on elimination rate as plasma data from the same subjects, as can be seen by comparison with Fig. 14.2 The picture has been obtained directly from a computer-plot.
TABLE 14.2

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dose (mg)</th>
<th>$C_{\text{max}}$ (mg/l)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>$t_{1/2}\text{el}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.Ba</td>
<td>50</td>
<td>3.01</td>
<td>2.0</td>
<td>7.2</td>
</tr>
<tr>
<td>J.Cl</td>
<td>50</td>
<td>3.32</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>J.Da</td>
<td>50</td>
<td>2.92</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>K.Ko</td>
<td>25</td>
<td>2.51</td>
<td>2.1</td>
<td>12.1</td>
</tr>
<tr>
<td>P.Pe</td>
<td>25</td>
<td>1.49</td>
<td>2.0</td>
<td>10.7</td>
</tr>
<tr>
<td>A.Ro</td>
<td>50</td>
<td>3.53</td>
<td>3.5</td>
<td>11.3</td>
</tr>
<tr>
<td>S.Si</td>
<td>50</td>
<td>1.83</td>
<td>1.2</td>
<td>3.0</td>
</tr>
<tr>
<td>F.So</td>
<td>50</td>
<td>2.32</td>
<td>5.5</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Some pharmacokinetic parameters describing red blood cell concentration of mefruside in humans after single oral 25 and 50 mg doses. The concentration vs. time data, taken from the same three subjects, from whom the plasma concentrations appeared in Fig. 14.2. Terminal half-lives, calculated by regression analysis of the final straight part of erythrocyte concentration curves in eight subjects are presented, together with maximum concentrations ($C_{\text{max}}$) and times of these maxima ($t_{\text{max}}$), in Table 14.2. No differences with the $t_{1/2}$ values resulting from plasma measurement were found, cf. Table 14.1.

**Urinary excretion of mefruside**

The urinary excretion rate plots of mefruside were parallel to the decay curves of plasma concentration in all subjects and no great dependence of urinary excretion rate upon either flow or pH of urine was observed. A typical plot is shown in Fig. 14.4. Only a very small proportion of the dose, ranging from 0.3-1.1% in eight subjects, mean 0.49%, had been excreted at infinite time. Detailed pharmacokinetic data are presented in Table 14.3. Renal plasma clearance of unchanged mefruside was very low, from 2.5-5.4 ml/min, see Table 14.3.

**Concentration of mefruside metabolites in plasma and red blood cells**

The concentration of 5-oxo-mefruside and its open acid analogue in plasma remained below the limit of detection of the analytical method, i.e. ca. 5 ng/ml. In red blood cells, 5-oxo-mefruside was measurable from the earliest sample, collected at 0.5 h, until at least 50 h after the single oral dose of mefruside. The open acid metabolite could be followed in red blood cells during 24-30 h, after which concentration became too low for accurate determination. Figure 14.5 shows red blood cell concentrations of the two metabolites in subject F.So, together with the concentrations of unchanged mefruside in plasma and red cells. The absorption pattern of
mefruside, being irregular in this subject, is closely reflected, after some delay, by the formation of metabolites. Maximum metabolite concentrations were reached ca. 10 h post-dose. The ratio of lactone concentration over open acid concentration was about 10 in the final part of the decay curves, although it had been lower, ca. 6-8 initially, see Fig. 14.5.

Similar data resulted from the erythrocyte concentrations in the three other subjects. Peak concentrations of the lactone metabolite were: 1.05 \( \mu g/ml \), 2.34 \( \mu g/ml \) and 0.83 \( \mu g/ml \) in the subjects H.Ba, J.Cl and S.Si, respectively. The corresponding maximum concentrations of the open acid metabolite were: 0.176 \( \mu g/ml \), 0.54 \( \mu g/ml \) and 0.11 \( \mu g/ml \), respectively, attained at 5-8 h after intake of mefruside. Terminal half-lives of the two metabolites could be calculated only approximately, especially that of the open acid compound, due to limited numbers of data points, but the \( t_{1/2} \) values were clearly longer than the half-life of mefruside itself, in each sub-
red blood cell and plasma concentration of mefruside and metabolites (µg/mL, log scale)

Subject F So, 23 yr, 65 kg
Dose 50 mg mefruside orally

Figure 14.5
Concentration-time curves of mefruside and main metabolites after oral administration of a single 50 mg dose of mefruside in man. 5-Oxo-mefruside (mefruside-lactone) and its open acid analogue were eliminated more slowly than the parent drug, as seen from the red blood cell concentration. The plasma concentration of the two metabolites was too low to be detectable.

ject. In the subjects H.Ba, J.Cl, S.Si and F.So the red blood cell concentration of 5-oxo-mefruside declined with t½ values of 13 h, 10 h, 14 h and 12.5 h, respectively.

Urinary excretion of 5-oxo-mefruside and its hydroxy carboxylic acid analogue.

Typical urinary excretion rate vs. time plots on semi-logarithmic scale are shown in Fig. 14.6. After intake of mefruside an abundant excretion of the two metabolites was seen, with high urinary excretion rates persisting during up to ca. 10-15 h after the dose. The cumulative urinary excretion (extrapolated to infinite time after actual analysis) amounted to mean values of 13.1% of dose and 46.2% of dose, respectively, for the lactone and the open acid metabolite. Detailed excretion data from four human subjects are given in Table 14.4. The terminal t½ of the lactone ranged from 10-14 h, and that of the open acid compound from 9-12.5 h.
### TABLE 14.3
Urinary excretion of unchanged mefruside after oral administration of single 25 or 50 mg doses in man

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dose (mg)</th>
<th>Time period measured (h)</th>
<th>Elimination half-life (h)</th>
<th>Cumulative excretion of mefruside during assay</th>
<th>Renal plasma clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>amount (mg)</td>
<td>% of dose</td>
</tr>
<tr>
<td>H.Ba</td>
<td>50</td>
<td>52.6</td>
<td>7.2</td>
<td>0.214</td>
<td>0.43</td>
</tr>
<tr>
<td>J.CI</td>
<td>50</td>
<td>13.5</td>
<td>3.4</td>
<td>0.130</td>
<td>0.26</td>
</tr>
<tr>
<td>J.Da</td>
<td>50</td>
<td>22.0</td>
<td>4.5</td>
<td>0.109</td>
<td>0.22</td>
</tr>
<tr>
<td>K.Ko</td>
<td>25</td>
<td>30.6</td>
<td>12.5</td>
<td>0.229</td>
<td>0.92</td>
</tr>
<tr>
<td>P.Pe</td>
<td>25</td>
<td>32</td>
<td>10.4</td>
<td>0.133</td>
<td>0.53</td>
</tr>
<tr>
<td>A.Ro</td>
<td>50</td>
<td>28.3</td>
<td>10.6</td>
<td>0.303</td>
<td>0.61</td>
</tr>
<tr>
<td>S.Si</td>
<td>50</td>
<td>18.9</td>
<td>3.1</td>
<td>0.135</td>
<td>0.27</td>
</tr>
<tr>
<td>F.So</td>
<td>50</td>
<td>24.7</td>
<td>6.2</td>
<td>0.145</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Mean: 0.49%

### TABLE 14.4
Urinary excretion of two oxidized metabolites of mefruside, viz. mefruside-lactone and carboxylic acid analogue, after oral administration of single 50 mg doses of mefruside to four healthy human subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time period measured (h)</th>
<th>Elimination half-life (h)</th>
<th>Cumulative excretion of lactone amount (mg)</th>
<th>% of dose</th>
<th>Cumulative excretion of open acid amount (mg)</th>
<th>% of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>during assay</td>
<td>at infinite time</td>
<td>during assay</td>
<td>at infinite time</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>amount (mg)</td>
<td>% of dose</td>
<td>amount (mg)</td>
<td>% of dose</td>
</tr>
<tr>
<td>H.Ba</td>
<td>55.4</td>
<td>14</td>
<td>6.79</td>
<td>13.6</td>
<td>7.25</td>
<td>14.5</td>
</tr>
<tr>
<td>J.CI</td>
<td>56.1</td>
<td>11.5</td>
<td>6.30</td>
<td>12.6</td>
<td>6.51</td>
<td>13.0</td>
</tr>
<tr>
<td>S.Si</td>
<td>55.4</td>
<td>10</td>
<td>5.84</td>
<td>11.7</td>
<td>5.99</td>
<td>12.0</td>
</tr>
<tr>
<td>F.So</td>
<td>55.5</td>
<td>10</td>
<td>6.21</td>
<td>12.4</td>
<td>6.37</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Mean: 13.1%

Calculated by extrapolation from plots of urinary excretion rate vs time
Figure 14.6
Urinary excretion rates of 5-oxo-mefruside, the hydroxy-carboxylic acid analogue thereof, and mefruside, plotted on semi-logarithmic scale against time. The subjects J.CI and F.So excreted 34.6% and 54% of dose respectively, as the open acid metabolite, and 13% and 12.7% of dose respectively, as the lactone metabolite. Only 0.28% and 0.30% of dose respectively was excreted as unchanged mefruside. The elimination half-lives of the metabolites of mefruside were approximately the same for each subject, ranging from 10-12 h.
Judged from the urinary excretion rate plots neither of the two metabolites responded much to changes in urine flow or urine pH, but the profiles of excretion of the two compounds were strikingly parallel to each other, see e.g. Fig. 14.6.

**Urinary excretion of conjugated metabolite**

After treatment of urine with glucuronidase an additional amount of the hydroxy carboxylic acid metabolite, corresponding to 5-15% of dose, was recovered over a ca. 55 h period (exact periods the same as indicated in Table 14.4). The figures were: 3.0 mg for subject H.Ba, 5.3 mg for subject J.Cl, 2.3 mg for subject S.Si and 6.9 mg for subject F.So.

In a pilot experiment it was checked that spontaneous hydrolysis of this conjugated metabolite did not occur in human urine at measurable rate. Thus, when urine samples obtained from two human subjects 4 h after ingestion of 50 mg of mefruside, were extracted at pH 2 immediately after voiding, values identical with those analysed after standing of urine for 1 day at room temperature were found. This implies that no in vitro liberation of the open acid metabolite of mefruside from its conjugated form had taken place.

**DISCUSSION AND CONCLUSIONS**

**Pharmacokinetic parameters**

The plasma concentration versus time curves of mefruside were well-fitted according to the appropriate model equations, see Fig. 14.2, and the terminal $t_{1/2}$ values could be measured accurately (Table 14.1). Estimates of the absorption rate constant, $K_a$, and the parameters $\alpha$ and $A$, were obtained with less precision in case of two compartment kinetics, because relatively few data points were available during the early distribution phase of each curve. These uncertainties did, however, not seriously influence estimation of plasma clearance and total volume of distribution, which was verified by calculating these parameters (divided by the bioavailability $F$) as $D/AUC$ and $D/\beta_AUC$, respectively, where $D$ represents the dose and $AUC$ the total area under the curve after extrapolation to infinite time. Plasma clearance values very close to the $k_{Cel}$ values presented in Table 14.1 were found. Similarly, the total volume of distribution by area, i.e. $V_{d\beta}$, was generally comparable in magnitude to the $V_{dss}$ values, although each $V_{d\beta}$ was systematically larger than the corresponding $V_{dss}$, which is in agreement with theoretical expectation, see, e.g., Wagner (1976). Conclusively, the important pharmacokinetic constants of Table 14.1 can be regarded as essentially model-independent parameters.
The large total volume of distribution, 314-518 liters equivalent to 5-7.5 liter/kg body weight, implies extensive tissue binding of mefruside. In rat experiments high levels of radioactivity were found, besides in red blood cells, in kidney and liver, already shortly after i.v. administration of mefruside (Duhm et al., 1967). So, it is reasonable to assume that the large $V_{dss}$ in man reflects binding to these tissues, and of course, as evidenced by our data, to red blood cells.

**Binding to red blood cells**

A linear distribution of mefruside between plasma and red blood cells was observed in vitro, and the rate of equilibration was unmeasurably rapid (Fleuren et al., 1979a; Chapter 4). Consequently, a constant ratio of mefruside concentration in plasma over its concentration in erythrocytes could have been expected for the present study. This turned out in general, see the Results section. Yet, at the top of the concentration time curves the ratios were somewhat lower than those present during the absorption and elimination phase, which could be due to competition of metabolites of mefruside for binding sites at higher concentration. This was in agreement with the observation that red blood cell binding of mefruside in vitro decreased with increasing concentration of 5-oxo-mefruside (Fleuren et al., 1979b; Chapter 5). However, as the red blood cell over plasma concentration ratio was constant in the terminal part of the plots, perfect agreement between terminal half-lives of mefruside concentration in plasma and red blood cells resulted. Therefore, red blood cell concentrations being ca. 30 times higher than those in plasma or, alternatively, whole blood concentrations constitute a comfortable means for measuring elimination half-lives of mefruside in man (see Table 14.2).

The observation that the open acid metabolite of mefruside was measurable in the red cell fraction of blood but not in plasma (Fig. 14.5) is surprising, as in vitro experiments indicated only a small degree of erythrocyte uptake of this anionic substance (Chapter 5; Fleuren et al., 1979b). In contrast with 5-oxo-mefruside, which accumulated in red blood cells with a concentration about 20 times higher than that in plasma, the concentration of the carboxylic acid metabolite in erythrocytes was only one-tenth of that in plasma. In view of the ability of fresh human plasma to convert the lactone to the open acid (Chapter 5), an artefact in the in vivo observation due to the experimental procedure cannot be excluded. So, it is possible that the apparent red cell concentration of the open acid metabolite resulted from in vitro hydrolysis of 5-oxo-mefruside in the small volume of plasma trapped between the red blood cells after centrifugation of the blood. (In turn, 5-oxo-mefruside must have come there by diffusion out of the red cells because no trace of it was detectable in plasma).

The nature of the binding of mefruside by red blood cells has not yet
been established. In order to go into some more detail, we incubated mefruside in fresh human blood, as described in Chapter 4, for 60 min at 37°C in the presence of varying amounts of acetazolamide, a drug most probably strongly bound to the carbonic anhydrase fraction of red blood cells (Maren et al., 1960; Wallace and Riegelman, 1977). At a mefruside whole blood concentration of 3.5 µg/ml, the following erythrocyte over plasma concentration ratios were found at acetazolamide concentrations of 0.0, 6.25, 26.8 and 53.5 µg/ml, respectively: 38.5, 27.8, 7.0 and 2.5. From this displacement by acetazolamide we conclude tentatively that binding to carbonic anhydrase plays a role in the accumulation of mefruside in red cells. This would be in accordance with the weak inhibition of red cell carbonic anhydrase activity by mefruside in vitro (Wilson and Kirkendall, 1970).

**Intersubject difference in elimination of mefruside**

The 4-fold difference between human subjects with regard to the elimination rate of mefruside, see Figs. 14.2 and 14.3 and Tables 14.1 and 14.2, must be explained by variation in the rate of metabolism, because renal clearance of unchanged drug accounts for removal of less than ca. 1% of the dose (Table 14.3). Intersubject variation of this magnitude has been observed with some other drugs, which are known to be metabolized by hepatic microsomal oxidation and it is believed that such differences are determined both by genetic and environmental factors (Sjöqvist et al., 1976a). At least, 60-65% of an oral dose of mefruside is oxidatively transformed in man, as judged from the sum of 5-oxo-mefruside and its open acid analogue in urine, see Table 14.4. Therefore, the kinetic behaviour of mefruside forms no exception as far as being caused by such oxidative processes.

**Relationship between pharmacokinetics and diuretic effect**

Many-fold interindividual differences in elimination half-life will be no advantage for a drug in general, because of concomitant variation in therapeutic response to be expected. The pharmacokinetic profile of mefruside, however, seemed to contradict a direct correlation with its biological action. Thus, especially in subjects with half-lives of only a few hours, an early cease of the diuretic effect of mefruside would be expected. On the contrary, a slow onset of water and salt diuresis has been found after single oral doses of 25 to 100 mg of mefruside in man, with peak effects observed at 4-12 h by Wilson and Kirkendall (1970) and at 6-12 h by Schwab and Immich (1967). Diuresis continued up to ca. 20-24 h after the dose (Brogden et al., 1974; Schwab and Immich, 1967; Wilson and Kirkendall, 1970).

Both 5-oxo-mefruside and the carboxylic acid metabolite of mefruside
possess intrinsic diuretic properties, similar to those of mefruside, which has been demonstrated by intravenous studies in rats (Meng and Kroneberg, 1967; cf. Chapter 15). The half-life of the two metabolites in humans, 10-14 h for each subject, would provide effective concentrations of diuretic agents for a longer time than that predictable from the variable kinetics of the parent drug. Therefore, the time course of blood concentration and urinary excretion rate of the oxidized metabolites of mefruside, see Fig. 14.6, appears to be much more compatible with the above duration of diuretic effects.

For many classes of drugs the concentration in plasma is believed to be an adequate measure of biological effects (McDevitt and Shand, 1975; Schneider and Ruiz-Torres, 1977; Sjöqvist et al., 1976b), although the actual relationship can sometimes be complex, as for the tricyclic antidepressants (Editorial, 1978). On the contrary, as discussed in Chapter 1, evidence is growing that the effects of diuretic drugs might correlate better with the urinary excretion rate of the active principles than with their plasma concentration. This is supported by the observation that each sulfonamide diuretic, from which data are available, is excreted into urine for a large part of the dose (Table 1.3, Chapter 1). Thus, both the time course and the extent of urinary excretion of 5-oxo-mefruside and its carboxylic acid analogue lead to the conclusion that these metabolites of mefruside are the active principles of this drug in man.

SUMMARY

Single oral 25 or 50 mg doses of mefruside were administered to eight healthy human subjects and plasma, red blood cells and urine were assayed during up to 50 h. In four of these subjects, the concentrations of two active metabolites of mefruside, 5-oxo-mefruside (mefruside-lactone) and its hydroxy carboxylic acid analogue, were also measured.

Mefruside was rapidly absorbed into plasma, with a mean half-life of 0.5 h. A 4-fold interindividual difference in elimination half-life was observed, ranging from 2.9-12.5 h. The decay was biphasic in five of the eight subjects. In the three others having the shortest $t\frac{1}{2}$ values initial distribution was not visible as a separate phase, so that a monophasic decay resulted. The variation in $t\frac{1}{2}$ was reflected in a large variation in total plasma clearance between subjects (22.5-129 l/h). The total volume of distribution ranged from 314-518 liters. Mefruside distributed instantaneously between plasma and red blood cells. The concentration in red blood cells was about 30 times higher that in plasma and the terminal decay was parallel to that of plasma concentration. Also the urinary excretion rate of mefruside
was parallel to the plasma concentration curves. Only a mean total of 0.49 % (± 0.30 %, S.D.) of dose was excreted in urine over infinite time as the unchanged compound.

Measurable concentrations of 5-oxo-mefruside were observed in the red blood cell fraction already in the first samples, i.e. at 0.5 h. The carboxylic acid metabolite reached red blood cell concentrations ca. 10 times lower than those of 5-oxo-mefruside. The plasma concentrations of these metabolites were not detectable, but high concentrations were found in urine. Total urinary excretion of the lactone metabolite over infinite time ranged from 12.0-14.5 % of dose (mean ± S.D., 13.0±1.1 %), and that of the open acid metabolite was 34.5-54.3 % of dose (mean ± S.D., 46.2±9.6 %). Another 5-15 % of the dose was recovered in urine as conjugate of the open acid metabolite.

Urinary excretion rate of both main metabolites became maximal between approximately 5-10 h after administration of mefruside, and the decrease thereafter showed much less intersubject variation in t½ than that observed with the parent compound. A mean t½ of 11.9 h (± 1.7 h, S.D.) was measured for the lactone, and a mean t½ of 10.5 h (± 1.6 h, S.D.) for the acid metabolite. Approximately the same t½ resulted from red blood cell measurement.

It was concluded that the urinary excretion profile of both metabolites corresponded very well with the clinically known duration of action of mefruside, and that the metabolites instead of the parent drug must be considered as the actual therapeutic principles in man.

REFERENCES


Fleuren, H L J, Thien, Th A, Verwey van Wissen, C P W, van Rossum, J M Absolute bioavailability of chlorthalidone in man a cross over study after intravenous and oral administration Europ J clin Pharmacol 15, 35-50 (1979c)
Maren, T H, Robinson, B, Palmer, R F Griffith, M E The binding of aromatic sulfon amides to erythrocytes Biochem Pharmacol 6, 21-46 (1960)
McDevitt, D G, Shand, D G Plasma concentrations and the time course of beta blockade due to propranolol Clin Pharmacol Ther 18, 708-713 (1975)
Meng, K, Kroneberg, G Pharmakologie vom N-(4'-chlor 3' sulfamoyl benzolsulfonyl)-N methyl-2-aminomethyl-2-methyl tetrahydrofuran, ein neuen diuretic wirkenden Verbindung Arzneim Forsch (Drug Res ) 17, 659 671 (1967)
Pütter, J, Schlossmann, K The degradation of mefruside The participation of a 'lactonase' in drug metabolism Biochim Biophys Acta 286, 186-188 (1972)
Schneider, J, Ruiz-Torres, A Digitalis effect and blood concentration Int J clin Pharmacol 15, 424 427 (1977)
Wagner, J G Linear pharmacokinetic equations allowing direct calculation of many needed pharmacokinetic parameters from the coefficients and exponents of polyexponential equations which have been fitted to the data J Pharmacokin Biopharm 4, 443 467 (1976)
INTRODUCTION

Studies on the metabolism of mefruside showed that the major metabolites were formed by oxidation of the 5-carbon atom of the tetrahydrofuran ring in both rats (Duhm et al., 1967) and humans (Fleuren et al., 1979; Chapter 14). The primary metabolite, the so-called 5-oxo-mefruside or mefruside-lactone, appeared to exist in equilibrium with its free acid analogue. Both species were found in urine, accounting together for ca. 35% of the dose in rats (Duhm et al., 1967) and for ca. 60% in man (Fleuren et al., 1979; Chapter 14). Meng and Kroneberg (1967) compared the pharmacodynamic properties of mefruside and these metabolites by intravenous studies in rats. No differences between the three compounds were found with regard to their diuretic effects, as evidenced by identical dose-response curves. These findings suggested that the diuretic activity of mefruside was attributable to its metabolites, because the parent drug itself was almost completely metabolized, with less than 1% of the dose being excreted unchanged into the urine (Duhm et al., 1967; Fleuren et al., 1979; Chapter 14). This would be in agreement with the observation that all sulfonamide diuretics show a considerable degree of urinary excretion of their active principles. So, as discussed in Chapter 1, evidence has arisen that in the first place the extent of urinary excretion of diuretic drugs determines their effectiveness rather than the height of their plasma concentration.

A detailed study of the interconversion of the two metabolites in vivo and in vitro was made by Schlossmann and Pütter (1973). The formation of the lactone from the free acid and, on the other hand, the hydrolysis of the lactone appeared to be un measurably slow in aqueous buffers in the pH 7-8 range. Rat plasma and rat liver microsomes, however, markedly catalyzed the interconversion of both metabolites. When the lactone was administered intravenously to dogs, it was rapidly transformed into the free acid and the same was found for the formation of the lactone from the free acid. Moreover, both the lactone and the free acid were detected in the plasma of dogs, already 3 minutes after an intravenous injection of mefruside (Schlossmann and Pütter, 1973).

In the light of these observations, the pure diuretic effect of the two metabolites could have been obscured, because it had been measured during
several hours (Meng and Kroneberg, 1967). This period should be sufficient by far for a considerable interconversion, and for this reason it was considered of interest to re-assess the diuretic properties of both metabolites. Evidently, an unequivocal conclusion on the intrinsic diuretic action of mefruside and the two metabolites can be drawn only when conversions by metabolism are ruled out.

The isolated perfused rat kidney seemed to fulfil this requirement, as the kidney has a relatively small drug metabolizing capacity compared with the liver (see e.g., Jones et al., 1979). Use was made of a perfusion technique developed by Slegers and coworkers (1979), by which the functional characteristics of the kidney, viz. reabsorption of sodium, potassium, glucose and water, are kept in good condition for at least one hour or longer. Although, as in any presently known isolated kidney preparation, loss of function occurs with time, the deterioration in the present technique is moderate, e.g. the mean fractional sodium reabsorption decreases only 0.35% per 5 minutes over a 90 minutes period (Slegers et al., 1979). This enabled us to study in this system the influence of drugs with a presumed diuretic activity on the water- and electrolyte excretion. For this purpose, the drug effects were compared with the values from control kidney perfusions, in which perfusate without drug had been used during an equal period. The study described in this chapter was aimed to give a first qualitative impression of the diuretic properties of mefruside and its metabolites, not distorted by in vivo metabolic factors.

MATERIALS AND METHODS

Drugs

Mefruside and 5-oxo-mefruside (mefruside-lactone) were obtained from Bayer (Wuppertal, G.F.R., courtesy of Dr. H.Horstmann) (Horstmann et al., 1967). The purity of mefruside was better than 99.5%, as described in Chapter 4. The mefruside-lactone sample contained about 1.25% of mefruside (see Chapter 5), but no attempt to separate the lactone from its parent drug on a preparative scale was made. The small percentage of contamination did not justify such an effort, in view of the qualitative comparison of this study aimed at. The same was judged to apply to a 3% portion of the open acid form in the crystalline lactone sample, found by analysis of a solution of the sample in phosphate buffer of pH 7.4, immediately after dissolution. Quantitative determination of the concentrations of the lactone and its open acid analogue was performed as described in Chapter 5. The open acid was formed from the lactone by dissolving the latter in 0.1 M aqueous sodium hydroxide. This solution was allowed to stand overnight prior to the perfusion experiment. Mefruside and both
metabolites were dissolved in the perfusion fluid (10 and 50 μg/ml) shortly before the kidney perfusions. The composition of the perfusion fluid is described in the next section.

**The isolated perfused rat kidney**

Male Wistar rats, weighing approximately 200-250 g, were used. A detailed description of the surgical procedure of the rat kidney isolation and the methods and equipment used in the perfusion has been given by Slegers and coworkers (1979). Some aspects, considered relevant with respect to the subject of the present investigation, will be dealt with in short. The perfusion method is in essence a modification of that published by Nishiitsutsuji-Uwo et al., 1967). However, a completely artificial perfusion fluid is used instead of a semi-synthetic one, and the perfusate is not recirculated.

After anaesthesia with pentobarbital (45 mg/100 mg body wt) and heparinization (500 I.U./rat), the right kidney and ureter are carefully dissected free of adhering tissue and the ureter is cannulated. A metal cannula, which is continuously flushed with the perfusion fluid, is introduced via the mesenteric artery into the renal artery and tied. To direct the venous outflow, a cannula made of glass is put into the vena cava with the tip at the place where the renal vein is entering and ligated. A schematic drawing of the ligatures made is presented in Fig. 15.1. After the kidney has been dissected completely free, it is carefully placed into a perspex perfusion

![Figure 15.1](image)

**Figure 15.1**

Schematic representation of the ligatures made in the isolated perfused rat kidney. The cannulas were entered at places marked by X. Reproduced, with permission, from Slegers and coworkers (1979).
chamber. The temperature of the perfusion fluid is kept at 37.5°C, just before entrance into the kidney. A constant perfusion flow of 15.1 ml/min is used, which results in a renal perfusion pressure of 120 mm Hg. The perfusion fluid has the following composition:

**Electrolytes (mM):** NaCl 124, KCl 5.2, CaCl₂ 1.0, MgCl₂ 0.3, NaHCO₃ 25, Na₂HPO₄ 0.84, and KH₂PO₄ 0.24

**Substrates (mM):** glucose 5, Na-pyruvate 1.99, Na-glutamate 1.2, Na-acetate 1.2, Na-propionate 2.1, and Na-lactate 1.0

**In addition:** urea 0.4 mM, alanine 5.0 mM, inosine 1.0 mM, insulin 4 I.U./liter, aldosterone 2 μg/liter, anti-diuretic hormone 10⁻² I.U./liter, and angiotensin II 15 ng/ml

**Plasma expander:** pluronic F108 (Wyandotte Chem. Corp., through BASF, Ludwigshafen, G.F.R.)

The pH of the perfusate is 7.4, when it is gassed with 5% CO₂ and 95% O₂ (v/v) and its osmolarity is 310 mOsmol/liter. The plasma expander pluronic F108 is a polymer of polyoxyethylene and polyoxypropylene with a molecular weight of 16250. It is electrically neutral, stable in solution, and biologically inert (Geyer, 1970). Physiological and morphological studies of Franke et al. (1975) have shown that better results are obtained during kidney perfusion with pluronic F108 than with dextran or haemacel.

Vitamin B₁₂ (cyanocobalamin) was added to the perfusion fluid, in a concentration of 20 mg/liter, as a marker for glomerular filtration rate. Previously, Slegers and coworkers (1979) had established that the ratio of the clearance of vitamin B₁₂ over the clearance of ¹⁴C-inulin was almost equal to unity in their system, viz. 1.025 ± 0.012 (mean ± S.E.M., n = 23) and that renal handling of sodium, potassium and water was not impaired by the concentration of vitamin B₁₂ used. The modified kidney preparation possesses, particularly under influence of the composition of the perfusate, a high reabsorbing capacity for electrolytes, glucose and water. E.g. over a 90 min. period, a fractional reabsorption of 96% for sodium has been achieved, whereas this lays in the order of 70% for potassium on an average (Slegers et al., 1979).

The kidney perfusions were performed in 90 minutes periods, divided into two sections. The first 45 minutes served as a control period (perfusion fluid alone). From 45 to 90 minutes the kidney was perfused with the perfusion fluid, which in addition contained one of the drugs in a concentration of 10 or 50 μg/ml. Urine samples were collected over 5 minutes periods and their volumes were measured by weighing. Vitamin B₁₂ concentrations in perfusate and urine were determined spectrophotometrically at 546 nm. A flame photometer was used to measure urinary sodium and potassium concentrations.
Calculation

Fractional reabsorption of sodium and potassium was calculated as:

\[
\% \text{ reabs.} = \frac{GFR \cdot c - U \cdot V}{GFR \cdot c} \cdot 100\%
\]

\[
GFR = \frac{\text{urine conc. vit. } B_{12}}{\text{perfusate conc. vit. } B_{12}} \cdot V
\]

where GFR = glomerular filtration rate; c and U concentrations of Na or K in perfusate and urine respectively, and V = urine flow rate

RESULTS

The urinary excretion of sodium and potassium together with urine flow in two typical experiments is shown in Fig. 15.2. The isolated rat kidney was perfused with fluid of normal composition during a control period of 45 minutes. Thereafter, perfusate to which drug had been added was used. Information on the performance of the individual kidneys was obtained from a number of renal parameters in the control period, viz. fractional reabsorption of sodium and potassium, and concentrating capacity. The data are presented in Table 15.1. By comparison with values found in a previous study by Siegers et al. (1979), it can be seen that the parameters in the present study fell in the normal range for those of such kidney preparations.

Diuretic effects were calculated as the percentual change of electrolyte excretion and urine formation with respect to a previous control period. The results are given in Table 15.2. Because every isolated kidney preparation loses salts and water with time, also when it has not been exposed to any diuretic drug, the changes obtained in perfusions without drug are also shown. Compared to the controls, only the perfusions with the open acid exhibited a consistent diuretic response. The lactone failed to be effective in one of the two experiments. Mefruside showed no increase in water and salt excretion at all.

DISCUSSION

The information on electrolyte excretion in control perfusions, in which no diuretic drug was added to the perfusate (Slegers et al., 1979), has been
Figure 15.2
Diuretic effects of the two main metabolites of mefruside in the isolated perfused rat kidney. Arrows indicate times, at which the normal perfusion medium without drug was replaced by one, in which the lactone or the free acid had been dissolved.

an essential pre-requisite for interpretation of our results. This provided a reference for the normal variation in losses of water and salt from the isolated kidney preparation. These always occur with time though to a variable extent (See Table 15.2). By comparing the diuretic parameters after the drug with the values from the control experiments, diuretic effects were observed for both metabolites of mefruside, but not for mefruside itself. The latter detail is surprising, because of the great structural similarity of the parent compound and the lactone. Apparently, the larger hydrophilicity of the metabolites as compared to mefruside (cf. Fig. 5.2, Chapter 5) favours diuretic action in this series of compounds. However, it cannot be excluded that higher concentrations of mefruside in the perfusion fluid would elicit any diuretic response. Moreover, the tacit assumption that drug metabolism would be insignificant in an isolated kidney preparation appeared to be not completely justified. Mefruside itself was
### TABLE 15.1

Kidney perfusion parameters in control period (40-45 min)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Drug concentration (µg/ml)</th>
<th>Fractional reabsorption (%)</th>
<th>Water conserving capacity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>sodium</td>
<td>potassium</td>
</tr>
<tr>
<td>no drug²</td>
<td>—</td>
<td>95.8 ± 2.4</td>
<td>89.8 ± 6.6</td>
</tr>
<tr>
<td>mefruside</td>
<td>10</td>
<td>96.3; 97.4</td>
<td>92.9; 94.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.9</td>
<td>91.0</td>
</tr>
<tr>
<td>lactone</td>
<td>10</td>
<td>96.3</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.6</td>
<td>94.3</td>
</tr>
<tr>
<td>carboxylic acid</td>
<td>10</td>
<td>96.4</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.1</td>
<td>88.8</td>
</tr>
</tbody>
</table>

¹Calculated as the ratio of the vitamin B₁₂ concentrations in urine and perfusate
²Mean values ± S.D., taken from Siegers et al. (1979)

### TABLE 15.2

Changes in diuresis, and excretion of Na and K after the control period¹

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Drug concentration (µg/ml)</th>
<th>% change</th>
<th>urine flow</th>
<th>ExNa</th>
<th>ExK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no drug²</td>
<td>—</td>
<td>29 ± 22.5</td>
<td>64 ± 52.5</td>
<td>122 ± 100</td>
<td></td>
</tr>
<tr>
<td>mefruside</td>
<td>10</td>
<td>10; 51</td>
<td>29; 119</td>
<td>13; 145</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>33</td>
<td>98</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td>lactone</td>
<td>10</td>
<td>186</td>
<td>581</td>
<td>372</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>38</td>
<td>115</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>carboxylic acid</td>
<td>10</td>
<td>98</td>
<td>312</td>
<td>249</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>152</td>
<td>445</td>
<td>714</td>
<td></td>
</tr>
</tbody>
</table>

¹Calculated by comparison of the average values during 45-70 min with those from the 25-45 min section of the control period
²Mean values ± S.D., taken from 7 perfusions (Slegers et al., 1979)
not metabolized during perfusion, judged from the absence of its main metabolites in pooled urine. Also the hydroxy carboxylic acid metabolite underwent the procedure apparently unchanged. On the contrary, after perfusion with 5-oxo-mefruside the open acid compound was present in urine in a concentration of ca. 10% of that of the lactone, indicating an in vitro conversion process. So, a possible influence of the open acid on the observations with the lactone cannot be disregarded at this stage.

A definite assessment of a difference in diuretic potency between the two metabolites of mefruside would demand perfusion experiments employing a broad concentration range. These studies would have to be performed at the expense of several kidneys at each concentration, due to biological variations in response (cf. Table 15.2). Therefore, any conclusion in this respect cannot be drawn at present and the positive evidence for intrinsic diuretic activity of the open acid metabolite of mefruside and also, but less unequivocally, of the lactone metabolite should be interpreted merely in a qualitative sense. These findings support the observations of the diuretic activity of both metabolites in the intact rat (Meng and Kroneberg, 1967), and thereby indicate the usefulness of a pharmacokinetic study of these compounds as the relevant diuretic principles of mefruside in vivo (cf. Chapter 14).

**SUMMARY**

Isolated rat kidneys were perfused with an artificial perfusion fluid containing mefruside, 5-oxo-mefruside or the hydroxy carboxylic acid analogue thereof. Diuretic effects were determined from the excretion of sodium and potassium and from water diuresis. The open acid compound exhibited a consistent diuretic response in two experiments. 5-Oxo-mefruside was active in one experiment, but not in the other. Mefruside failed to show diuretic action in all three experiments. These preliminary data can be regarded as support for the view that active metabolites of mefruside are the determinants of the in vivo diuretic effect of this drug.

**REFERENCES**


Dit proefschrift is gewijd aan de farmacokinetiek van sulfonamide-diuretica in de mens. Hoewel deze groep geneesmiddelen reeds in de zestiger jaren geïntroduceerd werd, was zij tot voor kort in farmacokinetisch opzicht nauwelijks onderzocht, mede door het ontbreken van adequate chemische analysemethoden.

Het hier beschreven onderzoek is dan ook in eerste instantie gericht geweest op het ontwikkelen van specifieke en gevoelige bepalingen van diuretica in biologische vloeistoffen. Om een aantal redenen werden speciaal chloortalidon en mefruside als onderwerp van studie gekozen, nl. a) beide middelen worden frequent toegepast in de medicamenteuze therapie ter bestrijding van hypertensie en edemen; b) vergeleken met de benzothiadiazines, waaraan chloortalidon en mefruside qua werking nauw verwant zijn, waren er bij aanvang van het onderzoek juist over deze verbindingen bijzonder weinig gegevens; c) tijdens vóóronderzoek bleek dat beide stoffen in vivo in hoge mate door rode bloedcellen worden opgenomen. Deze accumulatie in een gemakkelijk toegankelijk lichaamsweefsel bood mogelijkheden voor de modelmatige beschrijving van weefselbinding van geneesmiddelen in het algemeen.

Het proefschrift is onderverdeeld in vier secties. Sectie I bevat een algemene inleiding tot de sulfonamide-diuretica en een overzicht van hun farmacokinetische eigenschappen. Sectie II beschrijft de voor het kinetisch onderzoek aan chloortalidon en mefruside ontwikkelde analysemethoden. Sectie III behandelt de humane farmacokinetiek van chloortalidon (Hygroton®), en sectie IV die van mefruside (Baycaron®).

Sectie I: Diuretica en farmacokinetiek (hoofdstuk 1)

In HOOFDSTUK 1 worden de verschillende typen sulfonamide-diuretica gekarakteriseerd qua werking, aangrijpingspunten in de nier en wijze van transport vanuit de bloedbaan naar de urine. Het belang van het renale anion-transportsysteem bij de tubulaire secretie van diuretica wordt onderstreept, en de bijdragen van de processen van glomerulaire filtratie en tubulaire terugresorptie worden aangegeven. Na een inleiding tot de farmacokinetiek, waarin de mathematische verwerking van concentratie-tijdscuverten met behulp van lineaire en niet-lineaire compartimentenmodellen aan bod komt, en waarin belangrijke parameters als klaring en verdelingsvolume worden gedefinieerd, worden de farmacokinetische eigenschappen van sulfonamide-diuretica onderling vergeleken. Voor wat betreft
chloortalidon en mefruside wordt hierbij vooruitgelopen op de resultaten vermeld in de volgende hoofdstukken van dit proefschrift en voor wat de overige diuretica aangaat, wordt geput uit recente literatuur.

De potente lis-diuretica furosemide en bumetanide blijken zich in sterke mate te onderscheiden van de thiazides (waartoe ook chloortalidon, mefruside en andere heterocyclische verbindingen gerekend worden). Het totale verdelingsvolume van de twee eerstgenoemde stoffen is ongeveer 10-15 liter, hetgeen gering is ten opzichte van dat van de thiazides dat varieert van 100-500 liter. De grootte van het verdelingsvolume is duidelijk gecorreleerd met de mate van accumulatie van deze stoffen in nier- en leverweefsel en in rode bloedcellen. Het verschil in de totale klaring van sulfonamide-diuretica onderling is in het algemeen gering. Er wordt dan ook geconcludeerd dat de korte halfwaardetijd van verbindingen als furosemide en bumetanide louter het gevolg is van hun kleine verdelingsvolumina. Binnen de hele groep van sulfonamide-diuretica vertoont de duur van het diuretisch effect een sterke samenhang met de lengte van de halfwaardetijd. Literatuurgegevens en eigen experimenten wijzen erop dat niet de plasmaconcentratie van diuretica maar veeleer hun urinaire excretiesnelheid als maatstaf voor hun biologische werking beschouwd kan worden.

Sectie II: Analytische methoden (hoofdstukken 2-6)

In HOOFDSTUK 2 worden de principes van vloeistof-vloeistof extractie van sulfonamide-diuretica besproken op basis van de fysisch-chemische eigenschappen van deze verbindingen. Uit een overzicht van de analytische literatuur sedert 1960 blijkt dat de eerste bepalingen, gebaseerd op de Bratton-Marshall reactie, niet alleen als aspecifiek maar ook als ongevoelig gekenmerkt moeten worden. Van de huidige technieken, te weten dunne-laag chromatografie, hoge druk vloeistofchromatografie en gaschromatografie, is alleen de laatstgenoemde voorzien van voldoende gevoelige en specifieke detectoren om de uiterst lage plasmaconcentraties van de meeste sulfonamide-diuretica te kunnen meten. Hoge druk vloeistofchromatografie kan wel worden toegepast op de bepaling van deze verbindingen in urine, omdat de concentraties hierin een factor 50-100 maal hoger zijn dan de plasmaconcentraties. Voorafgaande aan gaschromatografische analyse van sulfonamide-diuretica is wegens hun polariteit derivatisering noodzakelijk. Principe en toepassingen van de in ons onderzoek gebruikte extractieve alkyleringstechniek worden besproken, alsmede de werking van de thermionische alkalizout detector, de z.g. stikstofdetector.

De gaschromatografische analyse van chloortalidon in plasma, urine en rode bloedcellen wordt beschreven in HOOFDSTUK 3. De gepermethy-
leerde derivaten van chloortalidon en zijn interne standaard worden gemeten door middel van een stikstofdetector. Hoeveelheden van de stof tot een benedengrens van 10 nanogram per biologisch monster kunnen bepaald worden met een nauwkeurigheid van 5% (relatieve standaarddeviatie).

Aangezien chloortalidon sterk in erythrocyten accumuleert, en het evenwicht van de concentraties in plasma en erythrocyten in vivo pas tussen de 10 en 15 uur na toediening van het geneesmiddel wordt bereikt, vindt ook na bloedafname transport van chloortalidon naar de erythrocyten plaats. Door dit in vitro proces kan de oorspronkelijke plasmaconcentratie met meer dan 50% verminderen. Een dergelijk artefact wordt vermeden door het bloedmonster onmiddellijk na de venepunctie te centrifugeren en het plasma snel te verwijderen. In verband met de hoge concentratie van chloortalidon in erythrocyten is eveneens de invloed van hemolyse op de plasmaconcentratiemetingen onderzocht.

**HOOFDSTUK 4** is gewijd aan de kwantitatieve bepaling van mefruside in lichaamsvloeistoffen. Extractieve methylering bij kamertemperatuur gedurende 5 minuten resulteert in een volledige omzetting van mefruside tot het dimethylderivaat. Met behulp van een analoge interne standaard wordt voor concentraties tussen 10 en 250 nanogram per milliliter een meetnauwkeurigheid verkregen van 4% (relatieve standaarddeviatie).

Ook mefruside wordt in sterke mate in erythrocyten opgeslagen, doch de hiermee samenhangende analytische complicaties zijn van andere aard dan in het vorige hoofdstuk beschreven. Het evenwicht van de plasma- en de rode bloedcelconcentraties van mefruside wordt praktisch momentaan ingesteld, waarschijnlijk wegens de grote lipofiliteit van mefruside. De ligging van dit evenwicht is echter temperatuur-afhankelijk. In bloed van 20°C zijn de plasmaconcentraties van mefruside ca. 35% lager dan in bloed van 37°C. Dit gegeven resulteert in een daling van de plasmaconcentraties van mefruside ten opzichte van de vivo waarden, indien bloed monsters onder routine-omstandigheden worden afgenomen. De meting van de biologische halfwaardetijd van mefruside wordt echter niet beïnvloed, omdat de plasmaconcentratie op reproduceerbare wijze met een constant percentage afneemt.

**HOOFDSTUK 5** behandelt de identificatie en de kwantitatieve analyse van twee hoofdmetabolieten van mefruside in de mens. Deze metabolieten, het lacton 5-oxo-mefruside en de open-zuur analoog daarvan kunnen afhankelijk van de pH van de oplossing in elkaar overgaan. Om ongewenste in vitro omzetting te vermijden werden optimale scheidingscondities bepaald door middel van een pH-partitie studie.

Bij een pH van 7.4 wordt het lacton geëxtraheerd zonder een spoor van

Door middel van gaschromatografie-massaspectrometrie werden beide stoffen als humane metaboolieten van mefruside geïdentificeerd. 5-Oxo-mefruside blijkt evenals mefruside te accumuleren in erythrocyten.

In HOOFDSTUK 6 wordt een potentiometrische verschiltitratiemethode beschreven ter bepaling van de pKa van chloortalidon. Deze methode is algemeen toepasbaar op farmaceutische verbindingen met een zeer geringe wateroplosbaarheid. Vier experimenten met chloortalidon leverden voor de schijnbare zuurdissociatieconstante in 0.1 M KCl een gemiddelde waarde van $9.24\pm0.02(\pm S.E.M.)$ bij $20^\circ C$. De thermodynamische dissociatieconstante werd, rekening houdend met ion-aktiviteitscoëfficiënten, bepaald op $pK_a=9.35$.

Sectie III: Farmacokinetiek van chloortalidon (hoofdstukken 7-13)

Het accent in HOOFDSTUK 7 ligt op de verdeling van chloortalidon over plasma en erythrocyten in vivo. Na orale toediening wordt de stof snel uit het maagdarmkanaal opgenomen en de plasmaconcentraties zijn maximaal tussen 1 en 3 uur. Het transport van chloortalidon naar de rode bloedcellen verloopt echter traag: piekconcentraties worden eerst tussen 10 en 15 uur na inname van de dosis waargenomen. De concentratie in rode bloedcellen is dan 50 tot 100 maal hoger dan die in plasma. De eliminatieduur van de erythrocytenconcentratie van chloortalidon is langer dan die van de plasmaconcentratie, gemiddeld respectievelijk 60 en 40 uur na doses van 100-200 mg aan tien proefpersonen. Een niet-lineair farmacokinetisch model was noodzakelijk om deze concentratie-tijdscurven te kunnen beschrijven. In dit model is het element van capaciteitsbeperkte binding van chloortalidon aan rode bloedcellen ingebouwd. De toepasbaarheid en de beperkingen van deze benadering worden besproken en vergeleken met de conventionele analyse volgens een lineair compartimenten model.

Ter ondersteuning van het in hoofdstuk 7 ontwikkelde farmacokinetische model, wordt in HOOFDSTUK 8 de binding van chloortalidon aan rode bloedcellen nader geanalyseerd door middel van in vitro experimenten. In het therapeutische concentratiegebied zijn voor intacte menselijke erythrocyten twee klassen van bindingsplaatsen aantoonaarbaar. De grotere compo-
nent heeft een totale bindingscapaciteit van ca. 150-200 μmol/liter en de schijnbare associatieconstante van chloortalidon is 1x10^6 liter/mol (0.9-1.1x10^6 l/mole). Voor de kleinere component werden waarden gevonden van 4-34 μmol/liter voor de totale bindingscapaciteit, en waarden van 0.7-7x10^7 liter/mol voor de schijnbare associatieconstante. De onzekerheid in de parameter schattingen van deze laatste component was echter groot. Derhalve kon de identiteit van deze bindingsplaats, die op grond van literatuurgegevens geassocieerd zou kunnen worden met het C-isoenzym van carboanhydrase, niet vastgesteld worden. De bindingsparameters van de grotere component daarentegen vertonen een goede overeenkomst met de bekende molaire concentratie van het B-isoenzym van carboanhydrase in humane erytrocyten en de affiniteit van chloortalidon voor het gezuiverde isoenzym.

In HOOFDSTUK 9 wordt de absolute biologische beschikbaarheid van chloortalidon tabletten vastgesteld aan de hand van een vergelijking met de intraveneuze (i.v.) toedieningsweg. Chloortalidon concentraties in plasma, rode bloedcellen, urine en faeces werden gemeten gedurende 100-200 uur na toediening van beide dosisvormen - in een sterkte van 50 mg - aan zeven proefpersonen. De eliminatie-halfwaardetijd (t½) van de plasmaconcentraties na i.v. dosering was 36.5 (±10.5 S.D.) uur, en die van de erytrocytenconcentraties 46.4 (±9.9 S.D.) uur. De gemiddelde t½ na orale toediening was langer: 44.1 (±9.6 S.D.) uur voor de plasmaconcentraties en 52.7 (±9.0 S.D.) uur voor de concentraties van chloortalidon in rode bloedcellen. Aangezien de kortere i.v.-halfwaardetijd niet gepaard ging met een toegenomen renale klaring, werd aangenomen dat een niet-renaal proces hierbij een rol speelde. Ook de som van de hoeveelheden, die als onveranderd chloortalidon in urine en faeces werden uitgescheiden, wijst sterk op de aanwezigheid van een niet-renaal en waarschijnlijk metabole eliminatierroute: na i.v. toediening wordt gemiddeld 65.4% (±8.6 S.D.) van de dosis in urine en 1.3-8% in faeces uitgescheiden. Na orale toediening: 43.8% (±8.5 S.D.) in urine en 17.5-31.2% in faeces. Het laatste gegeven is vrijwel uitsluitend toe te schrijven aan onvolledige resorptie van chloortalidon uit het maagdarmkanaal.

Voor de biologische beschikbaarheid van de orale dosis (F) wordt een gemiddelde waarde van 61% gevonden, indien de plasmaconcentratiemetingen als maatstaf worden genomen. Uit urinemetingen resulteert een F van 67%, en uit meting van de rode bloedcelconcentraties een F van 72%. Met behulp van berekeningen volgens het in hoofdstuk 7 beschreven niet-lineaire chloortalidon-model wordt aannemelijk gemaakt dat de schatting van F systematisch zo'n 10% te hoog uitvalt bij gebruik van rode bloedcelgegevens. Derhalve is het gemiddelde van de plasma- en urinemetingen, F = 64%, als de meest betrouwbare waarde te beschouwen.

283
De uitscheiding van chloortalidon in de urine wordt in detail besproken in HOOFDSTUK 10. Na orale toediening van enkelvoudige doses van 50 mg aan zes, 100 mg aan elf, en 200 mg aan zes proefpersonen verloopt in alle gevallen de renale excretiesnelheid van chloortalidon als functie van de tijd parallel aan de plasmaconcentratiecurve. Dit impliceert dat er geen concentratie-afhankelijke verzadiging van het tubulaire secretiesysteem van de nier is opgetreden. Overigens is de urinaire excretiesnelheid onderhevig aan grote fluctuaties over de tijd. Deze zijn echter niet toe te schrijven aan veranderingen in urine-flow of urine-pH, omdat de renale klaring van chloortalidon van de urine-flow niet, en van de urine-pH nauwelijks afhankelijk blijkt te zijn.

Het totale percentage van de dosis dat als onveranderd chloortalidon in de urine wordt uitgescheiden is dosis-afhankelijk: 44% (±7 S.D.) en 43% (±5 S.D.) na toediening van 50 en 100 mg respectievelijk, en 29% (±5 S.D.) na 200 mg. Een verminderd resorptiepercentage van de hoogste dosis werd op grond van faeces-analyses onwaarschijnlijk geacht. De andere mogelijke oorzaak, een verandering van de renale klaring, kon niet met zekerheid vastgesteld worden aan de hand van de hele groep proefpersonen, wegens de grote interindividuele variatie in deze parameter, bijv. 43-106 ml/min na de 100 mg dosis. Bij twee van de drie proefpersonen echter, die zowel 50, 100 als 200 mg chloortalidon hadden ontvangen, was de gemiddelde renale plasmaklaring van chloortalidon na de 200 mg hoeveelheid meer dan 25% lager dan die waargenomen na de 50 en 100 mg doses. Mogelijke implicaties van deze observatie worden bediskussieerd.

De uitscheiding via de galwegen als aandeel in de totale klaring van chloortalidon wordt behandeld in HOOFDSTUK 11. Zes galsteenpatiënten met ongestoorde nier- en leverfunctie ontvingen, 3-4 dagen na operatieve verwijdering van hun galblaas, een eenmalige dosis van 100-200 mg. De renale excretie van chloortalidon in deze groep was volledig vergelijkbaar met die gevonden in normale proefpersonen. De uitscheiding van het geneesmiddel in de gal was zeer gering, nl. 0.6-1.4% van de dosis gedurende 3-7 dagen. De conclusie luidt dat bij de mens galuitscheiding normaal gesproken geen belangrijke eliminatieroute voor onveranderd chloortalidon is.

Behandeling van gal- en urinemonsters met β-glucuronidase en arylsulfatase gaf geen verhoging van de daarin aanwezige chloortalidonconcentraties. Ook de open-zuur analoog van chloortalidon, die in de literatuur als metaboliet was gesuggereerd, bleek niet aantoonbaar te zijn.

HOOFDSTUK 12 heeft de relatie tussen de concentraties van chloortalidon in speeksel en plasma als onderwerp. Na toediening van een intraveuze dosis van 50-60 mg aan drie proefpersonen, werden plasma- en speekselmonsters verzameld gedurende 100 uur. De verhouding van speek-
De farmacokinetiek van chloortalidon bij chronische medicatie staat centraal in HOOFDSTUK 13. De proefopzet was de volgende: aan een patiënt met lichte hypertensie werden achtereenvolgens enkelvoudige en herhaalde doses van zowel 50 als 100 mg chloortalidon toegediend, over een totale tijdsduur van 90 dagen. Het experiment met de enkelvoudige doses was nodig ter verkrijging van kinetische basisparameters om de accumulatie van de concentraties na chronische toediening te kunnen voorspellen. Conform de concentratie-afhankelijke verdeling van chloortalidon over plasma en erythrocyten, zoals die beschreven is in de hoofdstukken 7 en 8, mocht verwacht worden dat de werkelijk gemeten concentraties tijdens de steady-state beduidend lager zouden zijn dan de volgens een lineaire kinetische analyse voorspelde waarden. De resulterende gemiddelde minimum steady-state concentraties \( C_{\text{min,ss}} \) waren: tijdens de periode waarin 50 mg chloortalidon per dag werd toegediend: plasma \( C_{\text{min,ss}} \) voorspeld 0.14 μg/ml, gevonden 0.12 μg/ml; erythrocyten \( C_{\text{min,ss}} \) voorspeld 15.5 μg/ml, gevonden 8.9 μg/ml. Tijdens de periode met 100 mg chloortalidon per dag: plasma \( C_{\text{min,ss}} \) voorspeld 0.34 μg/ml, gevonden 0.20 μg/ml; erythrocyten \( C_{\text{min,ss}} \) voorspeld 34.3 μg/ml, gevonden 17.1 μg/ml. Deze waarnemingen zijn volledig in overeenstemming met een capaciteitsbeperkte binding van chloortalidon aan erythrocyten.

Op het concentratieplateau bedroeg de 24-uurs urineuitscheiding van chloortalidon gemiddeld 38.2% (±5.1 S.D., \( n = 10 \)) van de dosis tijdens het doseringsschema van 50 mg per dag, en 47.5% (±5.3 S.D., \( n = 10 \)) tijdens toediening van 100 mg per dag. Mogelijke oorzaken voor dit significante verschil worden aangegeven.

**Sectie IV: Farmacokinetiek van mefruside (hoofdstukken 14 en 15)**

De opname in het lichaam, de verdeling ertover, de metabole omzetting en de renale uitscheiding van mefruside zijn de onderwerpen van de studie, die beschreven is in HOOFDSTUK 14. Deze processen werden geanalyseerd door middel van plasma-, rode bloedcel- en urineconcentratiemetingen na orale toediening van een dosis van 25 of 50 mg aan acht normale proefpersonen. Bij vier personen uit deze groep werden bovendien de concentraties van twee aktieve metabolieten van mefruside gemeten, namelijk
van 5-oxo-mefruside (mefruside-lacton) en van de hydroxy-carbonzure structuuranaloog daarvan. De resultaten laten zich als volgt samenvatten:

Mefruside wordt snel uit het maagdarmkanaal opgenomen met een gemiddelde absorptie-$t_1/2$ van 0.5 uur. De eliminatiesnelheid van mefruside verschilt sterk van individu tot individu: de $t_1/2$-waarden varieerden van 2.9-12.5 uur. Deze variatie weerspiegelde zich in de totale plasmaklaring, waarvoor waarden van 20-130 liter/uur werden gevonden. Het totale verdelingsvolume van mefruside daarentegen vertoonde minder spreiding (300-500 liter). Dit grote verdelingsvolume kan voor een belangrijk deel op rekening geschreven worden van de binding van mefruside aan erytrocyten. De erythrocytenconcentraties van de stof zijn gemiddeld 30 maal hoger dan de plasmaconcentraties. De instelling van het evenwicht tussen de concentraties in beide bloedfracties geschiedt praktisch momentaan, zodat uit de concentratiemetingen van mefruside in plasma en erythrocyten dezelfde eliminatie-halfwaardetijd resulteert. Ook de renale excretiesnelheid van mefruside als functie van de tijd verloopt in alle gevallen parallel aan de plasmacurve. De totale hoeveelheid die als onveranderd mefruside in de urine wordt uitgescheiden is echter gering, namelijk gemiddeld 0.5% ($\pm 0.3$ S.D.) van de dosis.

De metaboliet 5-oxo-mefruside was al kort na toediening van mefruside meetbaar in de rode bloedcellen. In plasma waren noch 5-oxo-mefruside noch de open-zuur metaboliet detecteerbaar, maar hoge concentraties van beide stoffen werden aangetroffen in urine. In totaal werd ca. 70% van de dosis als metaboliet in de urine teruggevonden, waarvan gemiddeld 46% (35-54%) als het hydroxy-carbonzuur, 13% (12-14.5%) als het lacton en 5-15% als conjugaat van het open zuur. De renale excretie van het lacton en het open zuur werd maximaal tussen 5-10 uur na toediening van mefruside. De eliminatie-halfwaardetijd van beide metabolieter was langer dan de $t_1/2$ van mefruside en er was nagenoeg geen interindividuele variatie in deze grootte: de $t_1/2$ van het lacton was gemiddeld 11.9 uur ($\pm 1.7$ S.D.), en de $t_1/2$ van de open zuur metaboliet 10.5 uur ($\pm 1.6$ S.D.).

Er wordt geconcludeerd dat het renale excretiepatroon van beide metabolieten zeer goed correspondeert met de diuretische werkdau van mefruside bij de mens en dat niet het mefruside zelf maar zijn metabolieten als de therapeutisch werkzame verbindingen beschouwd moeten worden.

**HOOFDSTUK 15** beschrijft enige inleidende onderzoeken naar de diuretische activiteit van de in hoofdstuk 14 aangetoonde hoofdmetabolieten van mefruside. Geïsoleerde rattenieren werden geperfusieerd met een kunstmatigeperfusievloeistof die hetzij mefruside, dan wel 5-oxo-mefruside of de hydroxy-carbonzure analoog daarvan bevatte. De open-zuur verhiding gaf een consistent diuretisch effect in twee experimenten, 5-oxo-mefruside was aktief in één van de twee proeven, maar mefruside was in
geen van de drie experimenten effektief. Deze voorlopige gegevens ondersteunen de bevinding dat aktieve metabolieten van mefruside het diuretisch effekt van deze stof bepalen.
The investigations were carried out in the Department of Pharmacology, University of Nijmegen, Nijmegen, The Netherlands, and were supported by a grant from the Dutch Foundation for Medical Research (FUNGO-ZWO).
DANKWOORD

Bij de voltooiing van dit proefschrift wil ik mijn dank betuigen aan iedereen die aan de totstandkoming ervan op enigerlei wijze een bijdrage heeft geleverd. De goede sfeer en de prettige contacten gedurende de afgelopen jaren op de afdeling Farmacologie en in het bijzonder de groep farmacokinetiek daarin hebben zeer stimulerend gewerkt en moeten op de eerste plaats vermeld worden.

Onmisbaar was de hulp van Mevr. C.P.W. Verwey-van Wissen, die op nauwgezette wijze én plezierig in de omgang talloze experimenten en ontelbare analyses uitvoerde, verder die van Mevr. E.L. Klok-Huyser, die de farmacokinetische modelberekeningen met inventiviteit ter hand nam, en die van Mevr. M.P.M. Klumpkens-Janssen, die het vele typewerk uitstekend verzorgde.

Veel dank ben ik verschuldigd aan de leden van de werkgroep farmacokinetiek: aan beide promotores Prof. Dr. J.M. van Rossum en Prof. Dr. C.A.M. van Ginneken, aan Drs. H.C.J. Ketelaars voor zijn spontaan gegeven medewerking, met name aan de massaspectrometrische analyses, aan Drs. P. Hekman en Dr. T.D. Yih voor de vele suggesties en discussies en aan Dr. J.M.G. van Kordelaar voor zijn gedegen kritiek terzake van het uiteindelijke manuscript. Vermelding verdienen tevens Mej. Drs. M.J.T. Peeters, en de Heren T.R. Boomkens en Drs. H.C.M. Koolen, die als doctoraalstudenten scheikunde een waardevol aandeel in het onderzoek hebben gehad; verder Mej. T.C.M. Marcelis en de analisten in opleiding: de Heer Th.J. Janssen en Mej. J.W. Hommersom.


Drs. Th.A. Thien (Interne Geneeskunde, Radboudziekenhuis, Nijmegen) ben ik zeer erkentelijk voor zijn enthousiaste bijdragen aan de in de hoofdstukken 9 en 11 van dit proefschrift beschreven onderzoeken, en Drs. W.H.L. Hoefnagels van dezelfde afdeling voor zijn medewerking aan de studie die weergegeven is in hoofdstuk 13. Mijn dank gaat verder uit naar Dr. S.H. de Bruin en medewerkers (Biofysische Chemie, Faculteit Wiskunde en Natuurwetenschappen, Katholieke Universiteit, Nijmegen) voor experimentele hulp en advies betreffende het onderzoek beschreven in hoofdstuk 6; naar Prof. Dr. J.F.G. Slegers en Mevr. M.T.G. Förster (Fysiologie, Medische Faculteit, Katholieke Universiteit, Nijmegen) voor het ter beschikking stellen van de nierperfusietechniek (hoofdstuk 15); naar Dr. J.C.M. Hafkenscheid (Klinisch-chemisch Laboratorium, Radboudzieken-


Op de laatste, maar zeker niet de minste plaats wil ik mijn ouders bedanken voor de steun over een veel langere periode dan die, welke het bewerken van dit proefschrift in beslag nam. In mijn vrouw Marion waardeer ik bijzonder haar inspanningen mij de gelegenheid te geven en de vele uren te gunnen om de uitkomsten van het onderzoek op schrift te kunnen stellen.

Alle betrokkenen zeg ik nogmaals hartelijk dank.

In augustus 1974 trad hij in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (ZWO) te Den Haag, en vanaf die tijd is hij werkzaam geweest op het Farmacologisch Laboratorium van de Katholieke Universiteit te Nijmegen. Hij heeft als lid van de werkgroep farmacokinetiek onder leiding van Prof. Dr. J.M. van Rossum en Prof. Dr. C.A.M. van Ginneken onderzoek verricht naar de analyse van geneesmiddelen in lichaamsvloeistoffen en naar het farmacokinetische gedrag van diuretica, voornamelijk bij de mens. Hij heeft het belangrijkste deel van deze onderzoekingen beschreven in dit proefschrift.

Hij is gehuwd en heeft een zoon en een dochter.

Uit gezamenlijk onderzoek zijn de volgende publicaties voortgekomen:


I

In tegenstelling tot hetgeen bij andere groepen geneesmiddelen het geval is, is de biologische werking van sulfonamide-diuretica niet zozeer van de hoogte van de plasmaconcentratie als wel van de mate van renale excretie van deze verbindingen afhankelijk.

Dit proefschrift.

II

Het natriuretisch effekt van mefruside bij de mens wordt niet door de stof zelf, maar door aktieve metabolierten veroorzaakt.

Dit proefschrift.

III

De gebruikelijke dosering van een aantal in Nederland veel voorgeschreven diuretica ter bestrijding van hoge bloeddruk kan, met behoud van de therapeutische werking en met vermindering van bijwerkingen, op z’n minst gehalveerd worden.


IV

Uit educatief oogpunt verdient het gebruik van tijdsconstantes in farmacokinetische studies de voorkeur boven de in de literatuur frequent gehanteerde snelheidsconstantes.
Het boek van Smith over de galexcretie van farmaca zou aan waarde hebben gewonnen indien bij de evaluatie van de humane studies de afwijkende fysiologie van galsteenpatiënten in aanmerking zou zijn genomen.


De massaspectrometrische bepalingsmethode van diazoxide volgens Sadee en medewerkers houdt geen rekening met het ontstaan van meerdere producten bij de derivatisering met diazomethaan.


De bevinding, dat een endogene inhibitor de binding van γ-aminoboterzuur aan hersenweefsel moduleert, onderstreept dat een bi- of multifasisch verloop van een Scatchard-plot niet zonder meer als bewijs voor het bestaan van twee of meer onafhankelijke bindingsplaatsen gehanteerd mag worden.


Evenals dit bijvoorbeeld voor een geschiedschrijver geldt, kan ook een biochemicus zich beter onthouden van een opzienbarend bewering, die bij nader onderzoek niet steekhoudend blijkt te zijn.


Gezien de klinische effekten van diazoxide, te weten water- en zoutretentie, en gelet op de experimentele condities van het onderzoek van Chrysant en Lavender, lijkt de waarneming van deze auteurs, dat toediening van diazoxide rechtstreeks in de arteria renalis zou leiden tot waterdiurese en zoutuitscheiding, een artefact.

De lever wordt ten onrechte nog te vaak bestudeerd als een systeem dat geïsoleerd kan worden van neuronale sturing vanuit de hersenen.


De poging van Campbell et al. het diureticum indapamide als anders dan andere sulfonamide-diureticate classificeren op grond van het renale excretiepatroon is ongefundeerd, omdat het bestaan van diuretisch werkzame metabolieten geenszins is uitgesloten.


Aan sommige plasmacurves valt geen $\tau$ vast te knopen.

Wachtkamers van ziekenhuizen en tandartsenpraktijken zouden minder afschrik wekken, indien bij de inrichting ervan meer kennis van de kleurenpsychologie was toegepast.

De spelling van het woord oligarchie lijkt wel verouderd. Beter ware: oligarchie.

H.L.J.M. FLEUREN

NIJMEGEN, 6 SEPTEMBER 1979