Monitoring

of occupational exposure to antineoplastic agents

Paul Sessink

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Contents

Abbreviations

AAS	atomic-absorption spectrophotometry
ALB	albumin
BEM	biological effect monitoring
BM	biological monitoring
CA	chromosomal aberrations
CAR	carboxyphosphamide
CP	cyclophosphamide
CREAT-U	urinary creatinine
ECD	electrochemical detection/electron-capture detection
EI	electron impact
EM	environmental monitoring
EMIT	enzyme-multiplied immunoassay technique
FBAL	*α*-fluoro-ß-alanine
FPIA	fluorescence polarization immunoassay
5FU	5-fluorouracil
GC	gas chromatography
GSH	glutathione
HPLC	high performance liquid chromatography
IP	iphosphamide
KCP	4-ketocyclophosphamide
MN	micronucleï
MS	mass spectrometry
MSD	mass-selective detection
MTX	methotrexate
NAD	nicotinamide adenine dinucleotide
NNM	nornitrogen mustard
NPD	nitrogen-phosphorus detection (see also TSD)
RBP	retinol-binding protein
SCE	sister-chromatid exchanges
SIM	selected-ion monitoring/single-ion monitoring
TFAA	trifluoroacetic anhydride
Tris	tris(hydroxymethyl)aminomethane
TRO	trophosphamide
TSD	thermionic-specific detector (see also NPD)
UV	ultraviolet

Chapter 1

General introduction

1.1 Antineoplastic agents

Antineoplastic agents[1] are widely used in the treatment of cancer and some non-neoplastic diseases [Black et al., 1990ab]. The cytotoxic activity may differ among the several antineoplastic agents. Depending on their mechanism of (anti-cancer) action, these drugs are subdivided in several categories such as alkylating agents, antibiotics, antimetabolites, and mitotic inhibitors (Table 1) [Black et al., 1990ab; Sorsa et al., 1985]. Alkylating agents express their activity by alkylating the DNA of tumor cells. Antibiotics interfere with the transcription of DNA. Antimetabolites block the synthesis of DNA and RNA. Mitotic inhibitors act on the mitotic mechanism necessary for karyokinesis. In therapy, antineoplastic agents are often used in a combination of drugs having different actions. The mechanism of action of some antineoplastic agents is still under investigation. Many antineoplastic agents interfere with DNA or DNA synthesis of tumor cells. Consequently, the proliferation of these cells is decreased. Antineoplastic agents are extremely active biological compounds. However, due to their in general non-selective mode of action, normal (non-tumor) cells may also be damaged resulting in toxic side-effects. Hence, workers handling these drugs, such as pharmacists, pharmacy technicians and nurses involved in the preparation and administration, and workers employed in the synthesis and production, may face certain health-risks.
Acute toxic side-effects of many antineoplastic agents such as irritation of skin, eyes and mucuous membranes, alopecia, nausea, vomiting and diarrhoea have frequently been observed in patients treated with these drugs. More severe toxic side-effects may occur in several organs and tissues such as bone marrow (leucopenia, anaemia, trombocytopenia), liver, bladder, kidney and lung [Black et al, 1990ab; Ladik et al., 1980; McDiarmid et al., 1988]. However, during occupational activities such as preparation and administration of these drugs, acute toxic side-effects have not been observed except for accidents in the course of which workers have been exposed to large amounts of spilled drugs.

[1] Synonyms: anticancer agents/drugs, antineoplastics drugs, antineoplastics, cytostatic agents/drugs, cytostatics, cytotoxic agents/drugs. In this thesis the synonyms are used interchangeably.

Apart from acute effects, special attention is given to the delayed toxic side-effects of antineoplastic agents such as mutagenicity, teratogenicity, and carcinogenicity [Sorsa et al., 1985].
Mutagenic effects have been observed in mammalian systems in vitro (animal and human cells) and in vivo (animals and humans) for almost all (alkylating) antineoplastic agents [IARC, 1981, 1986, 1987].
Teratogenic effects have been found in experimental animal studies for several, also non-alkylating, antineoplastic agents [IARC, 1981, 1986, 1987]. In some epidemiological studies, it is suggested that the increase in the number of spontaneous abortions and malformations in the offspring of nurses is caused by occupational exposure to antineoplastic agents [Hemminki et al., 1985; Selevan et al., 1985]. However, in a recent study no increased risks were found for miscarriages, malformations, low birth weight, or preterm birth among the offspring of nurses handling antineoplastic agents during pregnancy [Skov et al., 1992]. In one study liver damage in nurses is associated with occupational exposure to antineoplastic agents [Sotaniemi et al., 1983].
According to the International Agency for Research on Cancer, there is sufficient evidence for carcinogenicity of eight (alkylating) antineoplastic agents to humans (group 1) (Table 1) [IARC 1981, 1986, 1987]. These conclusions are based on epidemiological studies showing secondary tumors in cancer patients treated with these drugs and primary tumors in non-cancer patients treated with these drugs for other purposes [Baker et al., 1987; Greene et al., 1986]. In addition, several antineoplastic agents are carcinogenic in animal studies. They are qualified by the International Agency for Research on Cancer as probably or possibly carcinogenic to humans (group 2A and group 2B, respectively) (Table 1). A few antineoplastic agents (antimetabolites and mitotic inhibitors) are not classifiable to their carcinogenicity to humans by the International Agency for Research on Cancer (group 3) (Table 1).
A quantitative risk assessment of a toxic side-effect in relation to occupational exposure to antineoplastic agents has not been published (yet). Based on current scientific knowledge upon the mechanism of action of genotoxic carcinogens, among which almost all alkylating antineoplastic agents, the absence of a no-adverse-effect level is supposed. Therefore, exposure to these compounds should be avoided, and safety guidelines and protective measures such as wearing masks, gloves, gowns and special clothes and the use of vertical laminar airflow safety hoods were introduced to protect the workers when handling these drugs [ASHP, 1990; VIK, 1992; Skov, 1993]. For such compounds, an exposure corresponding to an acceptable small risk is strived for [Arboraad, 1992].

Table 1. Carcinogenicity of antineoplastic agents in relation to their mechanism of anti-cancer action[a].

antineoplastic agent (abbreviation/synonym)	CAS reg.no.	IARC evaluation[a]
alkylating agents		
N,N-bis(2-chloroethyl) 2-naphtylamine (chlornaphazine)	494-03-1	1
bischloroethyl nitrosourea (BCNU, carmustine)	154-93-8	2A
1,4-butanediol dimethanesulphonate (busulfan)	55-98-1	1
chlorambucil	305-03-3	1
1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, lomustine)	13010-47-4	2A
1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU, semustine)	13909-09-6	1
cisplatin	15663-27-1	2A
cyclophosphamide	50-18-0	1
dacarbazine	4342-03-4	2B
iphosphamide	3778-73-2	3
melphalan	148-82-3	1
nitrogen mustard (chlormethine, mustine)	51-75-2	2A
nitrogen mustard *N*-oxide	126 85-2	2B
streptozotocin	18883-66-4	2B
treosulphan	299-75-2	1
tris(1-aziridinyl)phosphine sulphide (thiotepa)	52-24-4	2A
antibiotics		
adriamycin (doxorubicin)	23214-92-8	2A
bleomycin	11056-06-7	2B
daunomycin (daunorubicin)	20830-81-3	2B
mitomycin C	50-07-7	2B
antimetabolites		
azathioprine	446-86-6	1
5-fluorouracil	51-21-8	3
6-mercaptopurin	50-44-2	3
methotrexate	59-05-2	3
mitotic inhibitors		
vinblastine	143-67-9	3
vincristine	2068-78-2	3
miscellaneous agent		
procarbazine	366-70-1	2A

[a] evaluation according to the IARC [IARC, 1981, 1986, 1987]
1: carcinogenic to humans 2A: probably carcinogenic to humans
2B: possibly carcinogenic to humans 3: not classifiable as to its carcinogenicity to humans

Occupational exposure to antineoplastic agents

In Dutch hospitals most antineoplastic agents are administered by infusion [IMS,1989]. During the preparation and administration of the drugs needles and broken ampules may result in pricks en cuts giving rise to a potential exposure. Contaminated vials and ampules are also a source of exposure. Contamination already present on the vials and ampules before preparation has started, will finally result in spreading out of the contamination, especially when the cleaning procedures are ineffective and inefficient. Besides, several studies have shown that the gloves used during preparation were permeated by the drugs, finally resulting in possible uptake of the drugs. During the preparation and administration of the drugs, some overpressure will result in the release of aerosols. In addition, urine, faeces, vomit, sweat and bedding and clothes of the patient may be contaminated with the drugs. This means not only a potential risk for the nursing staff but also for the workers in the laundry and internal transportservices and for family and friends of the patient. In addition, nurses prefer not to use protective measures such as special clothes, masks and gloves in order to treat the patient in a friendly way.

About fifteen years ago, it was shown that compared to the use of a horizontal flow hood, the use of a vertical-flow safety cabinet resulted in a decreased mutagenicity in the urine of the technicians preparing antineoplastic agents [Anderson et al., 1982]. Thereafter, several publications have shown the possible risks of hospital workers involved in the preparation and administration of antineoplastic agents. Consequently, protective measures were taken and safety guidelines were developed to protect the workers handling these drugs. Special attention was given to the effective use of protective clothes, masks and gloves. In addition procedures were improved and the vertical laminar airflow hood was introduced. In The Netherlands, the safety guidelines were introduced in 1986, amended in 1992 [IKN/IKO, 1986; VIK, 1992] and are reamended in 1996.

1.2 Methods for monitoring of occupational exposure to antineoplastic agents

In the previous section it is shown that antineoplastic agents not only destroy tumor cells but may also harm normal cells. This is inherent to the therapy with these drugs. For workers handling antineoplastic agents, exposure should be prevented because replacement of these agents by other less harmful drugs is not possible. A second way to reduce exposure is the use of closed systems. For several moments during drug handling this may be appropriate. However, total protection is not possible during treatment and care of patients. Finally, there is only one way to reduce exposure by using personal

protective measures combined with the application of guidelines. Despite the use of protective measures, one has to check whether there is still exposure in a qualitative and a quantitative manner. In occupational health several techniques are available to monitor exposure, dose or effect. Environmental monitoring (EM) and biological monitoring (BM) are used to measure environmental exposure and uptake (dose), respectively. Effects of drugs are assessed by biological effect monitoring (BEM) or health surveillance. Next, methods for monitoring of occupational exposure to antineoplastic agents hitherto used are briefly reviewed.

Environmental monitoring

Determination of air concentrations of antineoplastic agents (aerosols) is used for the quantification of external exposure to these drugs. After environmental air is sucked through a filter, the filters are extracted and analysed for the presence of antineoplastic agents.

Following this procedure and using high volume samplers, 5-fluorouracil (5FU) and cyclophosphamide (CP) were detected in the environment of pharmacy technicians and nurses involved in the preparation of antineoplastic agents [DeWerk Neal et al., 1983]. The samples were taken from three drug-preparation sites. 5FU was detected in nine samples (0.12-82.26 ng/m^3) and CP was present in one sample (370 ng/m^3). Methotrexate (MTX) and doxorubicine were not detected.

Release of 5FU from a horizontal air-flow hood was found by mimicking the preparation and administration of this drug [Kleinberg et al., 1981]. The mean concentration was 30 μg/m^3 (range: 0-70 μg/m^3).

No release of CP was found either in or outside of a vertical laminar flow hood or in a ward of a hospital during CP preparation and administration, respectively (detection limit: 0.05 μg/m^3) [Pyy et al. 1988; Sorsa et al., 1988, 1990]. However, a one year old HEPA filter of the hood was contaminated with CP (510 μg/m^2), showing release of CP.

In a comparable study no airborne 5FU was detected during drug preparation in the preparation area adjacent to the biological safety cabinet [McDiarmid et al., 1986].

High concentrations of CP were found in the air during the manufacturing process and drug production of CP in a pharmaceutical plant in Finland (stationary sampling: 0.1-810 μg/m^3; personal sampling: 0.6-190 μg/m^3) [Pyy et al., 1988; Sorsa et al., 1988, 1990]. The workers wore protective clothing, and gloves, and were supplied with air-respirators and encapsulating suits during all phases of potential high exposure. Besides, the workers were working in ventilated rooms and laminar flow hoods.

MTX has been determined in personal air samples of nurses involved in the preparation of CP, MTX, 5FU, adriamycin and cisplatin without any protection such as gloves, masks or a vertical laminar airflow hood [Friederich et al.,

1986]. MTX was detected in none of these samples (detection limit: 20 pmol/m^3.

Biological monitoring

Methods for biological monitoring can be divided in two groups: compound-selective methods and non-selective methods. For compound-selective methods, the amount or the concentration of a particular compound or its metabolites is determined by using sensitive chemico-analytical methods. For non-selective methods common properties of a group of chemicals are measured such as mutagenicity or electrophilicity. For biological monitoring of occupational exposure to antineoplastic agents the non-selective methods have been used very frequently. Some of the methods are discussed below.

Urinary mutagenicity assay

Some antineoplastic agents are alkylating compounds and express their cytotoxic activity by interaction with DNA resulting in a covalent bond. This interaction may result in the formation of mutations. These agents are mutagenic in several bacterial and eukaryotic test systems. An assay frequently used to detect mutagenic properties of chemical compounds is the so-called Ames-assay. Application of the Ames-assay on urine extracts of workers exposed to mutagenic compounds may indicate exposure to such compounds. However, no increase in mutagenicity does not mean no uptake of these compounds at all [Tuffnell et al., 1986]. Therefore, this approach should be considered as a signal test. In Table 2 an overview of studies is presented in which urinary mutagenicity is used as an indicator of exposure to antineoplastic agents. In several studies, an increase in urinary mutagenicity was observed. Other studies didn't show an increase. This assay is a compound non-selective method of biological monitoring which means that also other factors in addition to the exposure to antineoplastic agents may influence the results. In several studies an influence of smoking was observed [Barale et al., 1985; Bos et al., 1982; Breed et al., 1986; Courtois et al., 1987; Everson et al., 1985;]. The influence of smoking can be eliminated by the use of bacterial strains sensitive to antineoplastic agents but not to urinary mutagens due to smoking. However, it was also found that other factors are of influence such as diet and exposure to other environmental mutagens [Ames, 1983; Baker et al., 1982; Sasson et al., 1983]. Together, these factors cause a variation in background levels and it is questionable whether current exposure levels to antineoplastic agents will result in a significant increase in urinary mutagenicity [Friederich et al., 1986; Gibson et al., 1984]. This might be a possible explanation for the negative results in some studies. However, the urinary mutagenicity assay was suitable in a few studies to show the effect of protective measures such as masks and gloves. After introduction of these measures, the urinary mutagenicity was significantly

decreased [Falck et al., 1982]. It was also shown that exposure to antineoplastic agents could be significantly decreased if a horizontal laminar-flow hood is replaced by a vertical-flow biological safety cabinet [Anderson et al., 1982]. Horizontal laminar-flow hoods are normally used to protect the product while vertical-flow biological safety cabinets also protect the worker. In 1985, a large investigation was started in three Dutch hospitals to study the mutagenicity in urine of health care workers handling antineoplastic agents [Fransman et al., 1986]. No increase in urinary mutagenicity was found in the exposed group when compared to the control group. However, in 1981 and 1985 an increase in urinary mutagenicity was observed in nurses and pharmacy technicians working in Dutch hospitals [Bos et al., 1982; Breed et al., 1986]. The introduction of protective measures and safety guidelines are possibly the reason that in this study exposure to antineoplastic agents could no longer be detected.

Thioether assay

The determination of thioethers in urine is just like the urinary mutagenicity assay a non-selective method for biological monitoring. The assay is used as an indicator for exposure to (potential) electrophilic compounds. As mentioned before, some antineoplastic agents obtain electrophilic (alkylating) properties and may react with glutathione. Finally, mercapturic acids or thioethers can be formed and excreted in urine. Thus, an increase in thioether excretion is associated with exposure to (potential) alkylating compounds. Three papers have been published in which the thioether assay was applied to study occupational exposure to antineoplastic agents. In two studies an increase in thioethers was observed in the urine of nurses handling antineoplastic agents [Fransman et al., 1986; Jagun et al., 1982]. No safety measures were taken in one of these studies [Jagun et al., 1982]. In another study no increase in thioethers was observed in the urine of nurses. Most of them did not wear gloves and masks [Burgaz et al., 1988]. In summary, the thioether assay is a rather non sensitive method and the presence of background levels is strongly influenced by smoking. Hence, the thioether assay is less appropriate for BM of occupational exposure to antineoplastic agents.

Chemico-analytical methods

Determination of (metabolites of) antineoplastic agents in blood or urine due to occupational exposure belongs to the compound-selective methods of BM. Due to its chemical reactivity, the rather complex biotransformation pattern and an expected low exposure level, it is reasonable to assume low concentrations of (metabolites of) antineoplastic agents in urine or blood. Hence, sensitive methods are necessary. A few studies have been published.

Table 2. A literature overview of studies in which urinary mutagenicity has been used as a marker of exposure to antineoplastic agents.

reference	exposed group	result*
Falck et al. (1979)	nurses	+
Staiano et al. (1981)	pharmacists	-
Wilson et al. (1981)	pharmacy technicians	-
Anderson et al. (1982)	pharmacy personnel	+
Bos et al. (1982)	(smoking) nurses	+
Nguyen et al. (1982)	pharmacy technicians	+
Theiss et al. (1982)	pharmacists	+
Hoffman et al. (1983)	nurses	-
Kolmodin-Hedman et al. (1983)	nurses	-
	pharmacy technicians	+
Gibson et al. (1984)	nurses	-
Venitt et al. (1984)	nurses/pharmacists	-
Barale et al. (1985)	nurses	-
Cloak et al. (1985)	nurses	-
Everson et al. (1985)	nurses/pharmacists/pharmacy technicians	-
Benhamou et al. (1986)	nurses	+
Breed et al. (1986)	(smoking) nurses/pharmacy technicians	+
Connor et al. (1986)	pharmacy technicians	-
Fransman et al. (1986)	nurses/pharmacy technicians	-
Friederich et al. (1986)	nurses	-
Pohlová et al. (1986)	pilot plant workers, chemists, laboratory assistants	+
Stucker et al. (1986)	nurses	+
Courtois et al. (1987)	(non smoking) nurses	+
Caudell et al. (1988)	nurses	+
Poyen et al. (1988a)	nurses	-
Poyen et al. (1988b)	nurses/oncology personnel	-
Sorsa et al. (1988, 1990)	nurses/pharmacists	-
	process/production workers, laboratory technicians	-
Krepinsky et al. (1990)	nurses	-
Thiringer et al. (1991)	nurses	+
DeMéo et al. (1995)	mainly nurses and doctors	-

* +: urinary mutagenicity was significantly higher in the exposed group than in the control group
-: there was no significant difference in urine mutagenicity between the exposed group and the control group

In one study, CP was detected in the urine of two nurses involved in the preparation of CP for administration to patients during a 6-week test period [Hirst et al., 1984]. They did not take safety measures. Gas chromatography nitrogen-phosphorus detection (GC-NPD) and gas chromatography mass-selective detection (GC-MSD) was used for detection of CP. The mean

excretion of CP was 3.35 μg/day (range: 0.35-9.08 μg/day) in samples collected within 1-5 h after drug handling. In order to establish the route of exposure, the excretion of CP was studied after application of the compound on the skin of volunteers. It appeared that CP was excreted earlier in the urine of the nurses than in urine of the volunteers (18-24 h after drug application). It was concluded that the assumed uptake of CP in the nurses by inhalation occurs faster than dermal uptake in the volunteers.

Another study showed the excretion of CP in the urine of hospital workers despite the use of masks, gloves and a laminar air-flow hood. Once again CP was detected through the use of GC-MSD. The mean excretion of CP over 24 hrs was 1.6 μg (range: 0.7-2.5 μg) [Evelo et al., 1986].

Despite safety precautions platinum was found in the urine of exposed nurses and pharmacists handling cisplatin. Atomic-absorption spectrophotometry (AAS) was used as detection method. The mean excretion was 10.22 ng platinum/ml urine (range: 0.6-23.1 ng platinum/ml urine) [Venitt et al., 1984]. However, also in the urine of the control group of office workers platinum was found in equal amounts (mean: 8.9 ng platinum/ml urine; range: 2.6-15.0 ng platinum/ml urine).

Urine and blood samples have been taken from nurses involved in the preparation of CP, MTX, 5FU, adriamycin and cisplatin [Friederich et al., 1986]. None of the urine samples contained measurable amounts of cisplatin (detection limit: 2 μg/l). No MTX was detected in the nurses blood samples (detection limit: 2 nmol/l).

During the studies presented in this thesis other methods have been published. MTX has also been determined in the urine of nurses preparing MTX infusions and involved in care of patients [Mader et al., 1993]. The samples were determined by high performance liquid chromatography (HPLC) and ultraviolet (UV)-detection. The highest cumulative urinary excretion was observed in nurses preparing the MTX infusion, but traces of MTX were also detected in the urine of nurses engaged exclusively in the care of patients. The limit of detection was 4 ng MTX.

CP and iphosphamide (IP) were determined in urine samples of nurses and pharmacy personnel from eight hospitals [Ensslin et al., 1994b]. The analyses were performed by gas chromatography electron-capture detection (GC-ECD), detection limit 2.5 μg/24h urine. Despite standard safety precautions, including a vertical laminar airflow safety cabinet and gloves, CP and IP were detected in amounts ranging from 3.5 to 38 μg/24h (mean 11.4 μg/24h) and from 5 to 12.7 μg/24h (mean 9 μg/24h), respectively.

The platinum levels of nurses and hospital pharmacy personnel exposed to platinum containing antineoplastic agents were determined in 24h urine by voltametric analysis after UV photolysis [Ensslin et al., 1994a]. All study participants applied standard safety measures, including a vertical laminar airflow cabinet and gloves. Urinary platinum was significantly increased in a

few urine samples of a pharmacist and a pharmacy technician compared to a non-exposed control group. The limit of detection was 4 ng/l.

Biological effect monitoring

During the last 10 years, a quick development of new methods has taken place to detect so-called early, possibly reversible, biological effects of mutagenic and carcinogenic compounds (BEM parameters). Among these assays are the cytogenetic methods such as the analysis of chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) proliferation in blood lymphocytes. Cytogenetic methods are frequently used as marker of occupational exposure to antineoplastic agents.

Cytogenetic methods

CA in blood lymphocytes are made visible by cultivation of the lymphocytes followed by microscopic observation. Abnormalities are registered. SCE are observed if exchanges have taken place between sister-chromatids of chromosomes. The exchanges are only visible if both sister-chromatids can be distinguished from one another. A chromatid-break is supposed to be necessary for the formation of SCE. In the MN-test, lymphocytes are microscopically observed and scored on the presence of micronuclei. MN proliferation is seen as a direct result of damage to chromosomes. Table 3 shows an overview of studies performed with cytogenetic methods among hospital workers handling antineoplastic agents. In most studies no increase in cytogenetic effects was observed. Analogous to the mutagenicity assay, cytogenetic methods belong to the compound non-selective methods of BEM. This means that the effects found could also be caused by other factors. By using SCE and CA one should take into account that the effects measured are cumulative effects. So the effect measured at a particular moment is merely due to exposure in the past. Age is a confounder when using MN proliferation. MN are increased by age [Sorsa et al., 1988]. The causal relationship between cytogenetic effects and adverse health-effects is unknown. The usefulness of the cytogenetic methods is limited by a large variable background level. Besides, these methods are highly time consuming.

Pointmutations

Another technique is the assessment of mutation frequencies (HGPRT-locus) in lymphocytes as indicator of exposure to genotoxic compounds. Despite the use of gloves, masks and a vertical-flow safety cabinet, the number of pointmutations was increased in a group of nurses and pharmacists handling antineoplastic drugs when compared to their controls [Chrysostomou et al., 1984]. Mutation frequencies were also increased in a group of industrial workers employed in CP manufacturing and drug production compared to a non-exposed population [Hüttner et al., 1990].

Table 3. A literature overview of studies in which chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) proliferation in blood lymphocytes of hospital workers have been used as markers of occupational exposure to antineoplastic agents.

reference	parameter	result[a]
Norppa et al. (1980)	SCE	+
Waksvik et al. (1981)	CA and SCE	+
Szigeti et al. (1982)	SCE	-
Kolmodin-Hedman et al. (1983)	SCE	-
Stiller et al. (1983)	CA and SCE	-
Nikula et al. (1984)	CA	+
Barale et al. (1985)	SCE	-
Jordan et al. (1986)	SCE	-
Pohlová et al.[b] (1986)	CA and SCE	+
Stucker et al. (1986)	CA and SCE	-
Benhamou et al. (1988)	CA and SCE	-
Sorsa et al.[c] (1988, 1990)	CA and SCE	-
	MN	?[d]
Oestreicher et al. (1990)	CA	+
	SCE	-
Sarto et al. (1990)	CA and SCE	-
Krepinsky et al. (1990)	CA and SCE	-
Milkovic-Kraus et al. (1991)	CA and SCE	+
Cooke et al. (1991)	CA	-
Sardas et al. (1991)	SCE	+
Thiringer et al. (1991)	SCE	+
Grummt et al. (1993)	CA	+

[a] + : the parameter in question was significantly higher in the exposed group compared to the control group
- : there was no significant difference in the parameter in question between the exposed group and the control group

[b] only pilot plant workers, chemists and laboratory assistants

[c] pharmacists, nurses, process and production workers and laboratory technicians

[d] the results are inconclusive

Immunological effects
Many antineoplastic agents are immunosuppressive drugs. In one study immune functions were used as markers of exposure of nurses handling antineoplastic agents. No differences were observed between nurses and controls in respect of immune functions. It is supposed that the parameters are not sensitive [Lassila et al., 1980].

Conclusion
To assess occupational exposure to antineoplastic agents several methods have been developed and applied. Most studies concern compound non-selective methods for BM and BEM such as the urinary mutagenicity assay and the analysis of CA and SCE in peripheral blood lymphocytes. The disadvantage of these methods is that one can not prove beyond any doubt that the results found were caused by occupational exposure to antineoplastic agents. In addition, the sensitivity of these methods is low. In one study, no correlation was found between mutagenicity and CP concentration in urine [Evelo et al., 1986]. For occupational health services it is important to have the disposal of sensitive methods for BM of antineoplastic agents. In addition, these methods should also be specific for antineoplastic agents. In fact, methods should be developed for the determination of (metabolites of) antineoplastic agents in urine.

1.3 Objectives

The main question nowadays asked by many health care workers is: "Is there exposure and if so, what are the corresponding risks?" To answer the last question, it is necessary to measure the exposure to these drugs first. Second, exposure data are extrapolated by using dose-response relationships to establish risks in a qualitative and a quantitative manner. Till now, no quantitative risk asessments have been published to answer the last question. The problem is how to translate increases in non-specific parameters such as urinary mutagenicity, SCE and CA in peripheral blood lymphocytes into a quantitative risk. The results of the several studies show that the monitoring methods used nowadays are not sensitive and specific enough for the detection of the current exposure to antineoplastic drugs. Actually, at this moment adequate methods to determine occupational exposure to antineoplastic agents are lacking. The aim of the studies in this thesis is the development and validation of methods for monitoring of occupational exposure to antineoplastic agents. In this context, the following questions have to be answered:
1) Which (groups of) antineoplastic agent(s) should be selected for monitoring?
2) Are the developed methods applicable in situations in which hospital and other workers are exposed to antineoplastic agents?

1.4 Strategy

Selection of antineoplastic agents to be monitored
The aim of this study is to develop a small battery of compound selective EM and BM methods for the detection of exposure to antineoplastic agents. One of the first tasks is the selection of antineoplastic agents. Secondly, it is important to look at the pharmacokinetics of these drugs. Thirdly, it is necessary to look at the analytical possibilities to determine these antineoplastic agents or their metabolites in low concentrations in combination with their chemical stability.

Amount of antineoplastic agents used in The Netherlands
There are now approximately 50 antineoplastic agents commercially available for the treatment of cancer. Nitrogen mustard, the first drug used in cancer therapy was introduced in the late 40's. Recently taxol and taxotere have been introduced. It is expected that new drugs will be developed in the future because chemotherapy, in addition to surgery and radiotherapy, will remain an important approach in the treatment of (non)-neoplastic diseases. Moreover, new (immuno)chemotherapies will be developed based on further clarification of the mechanism of carcinogenesis.
Data on the use of antineoplastic agents in The Netherlands over 1986-1988, the starting period of the investigations presented in this thesis, showed a large difference between the use in hospitals and outside hospitals [IMS, 1989]. In hospitals, antineoplastic agents are mostly prepared for parenteral administration (infusion). Outside the hospitals, antineoplastic agents are mostly prescribed as tablets (oral administration). In Dutch hospitals, 5FU, CP and IP contributed to about 40% of the parenterally applied antineoplastic agents (wt basis) (Table 4).
The large number of clinically applied (combinations of) antineoplastic agents makes it necessary to look for one or more markers to be representative for the total group of antineoplastic agents. Based on the above information, 5FU, CP and IP were selected as antineoplastic agents to be monitored in the studies presented in this thesis. During several studies it was found that the amount of MTX was large enough to add this compound to the list of drugs to be monitored. The chemical structures of these compounds are presented in Figure 1.

Pharmacokinetics
Much information is available in literature about the biotransformation of CP, IP, 5FU and MTX in both man and animal in therapeutic doses [IARC, 1981; Sladek, 1985; Black et al., 1990a]. Except for MTX, the biotransformation pattern is rather complex. No studies are available using the relatively low doses to be expected during occupational exposure. The drugs or their

metabolites are mainly excreted in urine.

Chemico-analytical possibilities and stability

Most analytical methods are based on the determination of the parent compound in blood of treated patients and animals. Less attention is given to the determination of the parent compound or their metabolites in urine. Compounds or their metabolites in urine should be concentrated to obtain enough compound for detection. A careful and selective clean-up procedure may finally influence the results positively.

GC analysis in combination with ECD, NPD and MSD (SIM) is frequently used for the determination of CP and IP [Ensslin et al., 1994b]. Before analysis the parent drugs are derivatized with trifluoracetic anhydride (TFAA). The highest sensitivity is expected from GC-MS(D).

Analysis of 5FU is more problematic because hardly any 5FU is excreted in urine of 5FU-treated patients [Bernadou et al., 1985; IARC, 1981]. Therefore the metabolite α-fluoro-ß-alanine (FBAL), an amino acid, was choosen which appeared to be excreted in the urine of a 5FU-treated patient in relatively large amounts [Bernadou et al., 1985]. A routine derivatization method, frequently used for GC determination of amino acids was used [Schallenberg et al., 1955].

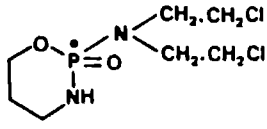
Table 4. Amounts of 5FU, CP, and IP parenterally administered (infusion) in Dutch hospitals over 1986-1988 [IMS, 1989].

antineoplastic agent	1986			1987			1988		
	kg	units	%*	kg	units	%*	kg	units	%*
5FU	22 2	72,400	17	17 9	68,500	16	28 8	114,500	20
CP	13 8	25,600	10	16 8	25,600	10	14 9	26,200	10
IP	12 0	14,500	9	12 6	12,200	9	12.3	9,300	8
total	48 0	112,500	36	47.3	106,300	35	56.0	150,000	38

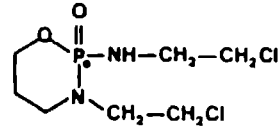
* as percentage of the total amount of parenterally administered drugs

Analysis of MTX in blood is frequently used in patients to prevent toxic side-effects after high-dose administration of this drug. HPLC with UV detection, fluorescence polarization immunoassay (FPIA) and enzyme-multiplied immunoassay technique (EMIT) are most frequently used as methods for monitoring [Tjaden et al., 1990]. Preference is given to FPIA and EMIT because of less interference with other compounds and consequently high sensitivity [Pesce et al., 1986]. Solid-phase extraction is an appropriate technique for concentration of MTX out of urine.

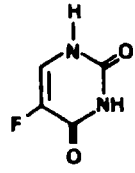
Fig. 1. Chemical structures of CP, IP, 5FU and MTX.



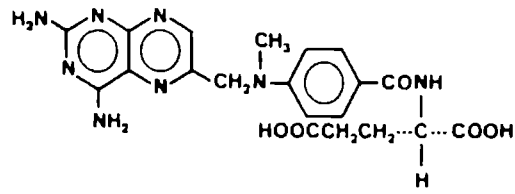
cyclophosphamide (CP)



iphosphamide (IP)



5-fluorouracil (5FU)



methotrexate (MTX)

Environmental monitoring

Several approximations are available to estimate concentrations or amounts of antineoplastic agents in the work environment. In this thesis the following aspects are presented and discussed.

1) Development and validation of methods for the determination of concentrations of antineoplastic agents in the air (aerosols and particles).
2) Development and validation of wipe-tests for the determination of environmental contamination and possible skin exposure and uptake.
3) Quantification of antineoplastic agents on the skin by skin contamination tests with gloves.

Biological monitoring
For the estimation of the internal dose, BM is choosen as approach and urine as matrix. Finally, the BM results in combination with toxicokinetic investigations in animals will be used to estimate the internal exposure (dose) in occupationally exposed workers. Such information may play an important role in eventual risk assessment procedures. In addition, other information should be available such as dose-response relations in animals and in patients treated with antineoplastic agents [Baker et al., 1987; Greene et al., 1986; Schmähl et al., 1979]. Afterwards, the efficiency of the protective measures can be checked with the aid of the developed analytical methods.
For BM, an analytical method developed by Evelo et al. for the determination of CP is considered to be most appropriate as starting point [Evelo et al., 1986]. This method is further optimised to be suitable for BM. Several toxicokinetic aspects are of importance for adequate application of BM and should be further investigated. Many studies have been undertaken to the kinetics of CP in the clinical setting. However, the question is how the kinetics of CP will be at low doses as expected during occupational activities. To answer this question, the uptake and the kinetics of low dose CP administration by other exposure routes are studied in experimental animals.

1.5 Outline of the thesis

This thesis describes the development and validation of methods for monitoring of occupational exposure to antineoplastic agents.
In **chapter 1** a general introduction to the subject is given. Attention is paid to the use of antineoplastic agents for the treatment of several diseases, their pharmacological activity, mechanism of action, toxicity, health risks and protective measures and safety precautions taken to protect the workers handling these drugs. Methods frequently used for monitoring of occupational exposure to antineoplastic agents are summarized and their usefulness is discussed. Finally the objectives of the thesis and the strategy is outlined and literature references are given.
Chapter 2 shows some developed and applied methods for monitoring occupational exposure to antineoplastic agents in several occupational settings. All methods are based on CP, IP, 5FU and MTX, the four most frequently used antineoplastic agents for parenteral administration (infusion) in The Netherlands over 1986-1988.
Some biological effects in workers exposed to antineoplastic agents are described in **chapter 3**. In a study, the analysis of CA in blood lymphocytes as BEM parameter is compared with the BM parameter CP in urine. In another study, the influence of antineoplastic agents upon renal effect parameters in workers exposed to antineoplastic agents is investigated.

Variation in uptake, biotransformation and excretion of CP, studied under several in vitro conditions (human and rat liver preparations) and in vivo conditions (rat), are presented in **chapter 4**.
Finally, a quantitative cancer risk assessment for health care workers exposed to CP is presented in **chapter 5**.
In **chapter 6** the results of the research project are summarized and discussed.

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Chapter 2

Development and validation of methods for environmental and biological monitoring

2.1

Detection of contamination with antineoplastic agents in a hospital pharmacy department

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Abstract

The contamination with fluorouracil (5FU), cyclophosphamide (CP) and methotrexate (MTX) was studied in a hospital pharmacy department where these drugs were prepared. In the preparation room, air samples were taken before and during preparation of the drugs. MTX was detected in one sample which was collected during preparation (0.3 $\mu g/m^3$). Spot samples were taken in the vertical laminar air-flow safety hood before and after preparation of the drugs and after cleaning of the hood. Contamination of the laminar air-flow hood was: CP: 1-160 ng/cm^2; 5FU: 10-62 ng/cm^2 and MTX 2-623 ng/cm^2). Spot samples from the floor in front of and beneath the laminar air-flow hood showed contamination with especially 5FU (48-236 $\mu g/m^2$). The gloves used during preparation of the drugs were contaminated mainly with 5FU (5-980 ng/cm^2). Urine samples from two workers involved in the preparation of the drugs were analysed for unmetabolized CP; it was not detected. Although no uptake of CP was established, it is shown that the methods for measurement of CP, 5FU and MTX in the preparation room are applicable for the control of occupational exposure to these drugs.

Introduction

The toxic side-effects of antineoplastic agents have been described extensively [1]. Much attention has been paid to the carcinogenic, mutagenic and teratogenic properties of these compounds, which are related to the interaction of the drugs with DNA, RNA and proteins.
Several investigators have studied occupational exposure to cytostatic drugs

by determining urinary thioethers [2,3], urinary mutagens [4-26], chromosomal abberations and sister chromatid exchanges in lymphocytes [14-16,24-32] or by measurement of cytostatic drugs like CP in urine [33,34]. In many cases the measurements demonstrated occupational exposure of hospital personnel [2,17-29,33-35].
To a lesser extent attention was paid to the question how the persons were exposed. It was suggested that during preparation antineoplastic drugs were absorbed by inhalation and dermal penetration [33,36,37].
To prevent occupational exposure to cytostatic drugs in the Netherlands, but also in other countries, special safety guidelines were introduced [38,39]. However, little is known about the effectiveness of the advised measures because methods to check these measures were not or scarcely developed. In addition, there is still great concern about the health risks due to exposure to cytostatic drugs among hospital workers [40].
For these reasons, it is important to be able to measure the spill and distribution of cytostatic drugs when handling these agents. Not only the contamination of the working environment but also the uptake of cytostatic drugs in hospital workers should be measured in order to identify risky actions and circumstances. Therefore, we have studied the contamination with cytostatic drugs of the pharmacy department of a Dutch hospital where these drugs were prepared. Because 5FU, CP and MTX were the most applied cytostatic drugs in this hospital, these substances were measured in air samples, and in spot samples taken in the laminar air-flow hood and from the floor in order to validate the applied methods and to study the efficiency of the guidelines and precautions used. Also contamination with cytostatic drugs of the gloves that were used was investigated. Finally, we have studied the uptake of CP by measurement of unmetabolized CP in urine.

Methods

Study design

Air, wipe and urine samples were taken on four separate days during two weeks of preparation (twice on a Monday and twice on a Friday). 2 Technicians were involved in the preparation; worker A on the first Monday (day M1) and Friday (day F1) and worker B on the second Monday (day M2) and Friday (day F2). The way of preparation of the drugs and the working methods of the two workers were observed and described. The drugs were prepared in a laminar air-flow hood (Class II, model DLF BSS 6, Clean Air, Woerden, the Netherlands).
In Table 1 an overview of the amounts of CP, 5FU and MTX prepared during this study is given. CP, 5FU and MTX contributed 96% of the drugs prepared.

Table 1. Amounts of CP, 5FU and MTX prepared during this study (mg).

day	worker	CP[a]	5FU[a]	MTX[a]	total[b]
M1	A	2,900 (60)	1,500 (31)	64 (1)	92
F1	A	1,500 (33)	2,750 (61)	140 (3)	97
M2	B	np[c] (-)	1,000 (90)	80 (7)	97
F2	B	np (-)	1,750 (95)	70 (4)	99
total of 4 days		4,400 (36)	7,000 (57)	354 (3)	96

[a] number in parentheses: as percentage (wt/wt) of all drugs prepared
[b] sum of CP, 5FU and MTX as percentage (wt/wt) of all drugs prepared
[c] np: no preparation

Air sampling

Before and during preparation of the drugs, air samples were taken approximately 50 cm from the worker and the laminar air-flow hood. The samples were collected on a glass microfibre filter with 150 mm diameter and 1.6 μm pore size (Whatman GF/A, Maidstone, UK) using a high-volume sampler (type Gromoz, Department of Air Pollution, Wageningen Agriculture University, Wageningen, the Netherlands). The suction flow was about 900-1,000 l/min. After sampling, the filters were packed in a glass pot and 50 ml of a sodium hydroxide solution (0.03 mol/l) was added. After sonification during 90 min, the extracts were centrifuged and analysed by high pressure liquid chromatography (HPLC) and gas chromatography (GC).

Wipe tests in the laminar air-flow hood

Before and after preparation of the drugs and after cleaning of the laminar air-flow hood at the end of the day, spot samples (1-6) were taken from several places for the detection of contamination of the laminar air-flow hood with cytostatic drugs. Spot samples 7 and 8 were added on days F1, M2 and F2 and spot samples 9 and 10 were added on days M2 and F2. A description of the spots is given in Table 2.

Two tissues (Kleenex professional wipes, 20 x 21 cm, Kimberly-Clark Corporation, Koblenz, Germany) were moisturized with 5 ml of a sodium hydroxide solution (0.03 mol/l) and the spots were swept clean. The tissues were put in a glass pot and 45 ml of a sodium hydroxide solution (0.03 mol/l) were added. After sonification during 90 min the extracts were centrifuged and analysed by HPLC and GC. Routine cleaning of the laminar air-flow hood occurred by using a gauze pad which was soaked in an alcohol solution.

Table 2. Description of the spots in the laminar air-flow hood.

spot	description*
1-4	working tray (100)
5-6	top side aerofoil (100)
7	top side aerofoil (300)
8	front grill of the working tray (300)
9	back side aerofoil (500)
10	inside viewing screen (8500)

* number in parentheses is the area swept clean (cm^2)

Wipe tests of the floor

Spot samples of the floor were taken once before preparation on day F1. They were taken from the floor in front of and beneath the laminar air-flow hood (11-16), in the centre (17) and at the entrance (outside) of the preparation room (18).

On each spot (approximately 0.5 m^2), 10 ml of a sodium hydroxide solution (0.03 mol/l) were pipetted. The spots were swept clean with four tissues (see above). The tissues were put together in a glass pot and 90 ml of a sodium hydroxide solution (0.03 mol/l) were added. After sonification during 90 min the extracts were centrifuged and analysed by HPLC and GC.

Glove contamination

During preparation of the drugs gloves were worn (Ansell Gammex pre-powdered sterile latex hypoallergenic surgical gloves, 0.52 mm thickness, Ansell Medical, Münich, Germany). Four pairs were used on day M1, three pairs on day F1, two pairs on day M2 and one pair on day F2.

After preparation of the drugs, left and right glove were collected separately and put in glass pots with the possibly contaminated side outside. 100 ml Of a sodium hydroxide solution (0.03 mol/l) were added and the samples were shaken during 120 min. The extracts were centrifuged and analysed by HPLC and GC.

Urine sampling

Total urine was collected in several portions during the period of 24 h after preparation of the drugs. They were stored at -20°C until sample preparation.

Chemicals

CP (Endoxan; purity >97%) and iphosphamide (IP) (Holoxan; purity >97%) were purchased from ASTA-Pharma (Bielefeld, Germany). Trifluoroacetic anhydride (purity >98%) was obtained from Johnson Mattey (Karlsruhe, Germany). 5FU (purity 99%) was purchased from Janssen Chimica (Beerse, Belgium) and MTX (purity 99.7%) was donated by Pharmachemie (Haarlem, the Netherlands). All other chemicals were of the highest purity obtainable. Distilled water was used.

For preparation CP (Endoxan in vials of 100, 200, 500 and 1,000 mg) was purchased from ASTA-Pharma, 5FU (Fluorouracil in ampules of 250 mg/5 ml) from Hoffmann-La Roche (Mijdrecht, the Netherlands) and methotrexate (Ledertrexate in vials of 5 and 50 mg/2 ml) from Lederle (Etten-Leur, the Netherlands), respectively.

HPLC analysis

5FU and MTX analyses were conducted on a reversed-phase HPLC system (Pye Unicam LC3-XP, Pye Unicam Ltd., Cambridge, UK). Aliquots of 20 μl of the centrifuged extracts were injected onto a 150 x 4.6 mm i.d., Nucleosil RP 18 column with 5 μm particles (Macherey-Nagel, Düren, Germany). The column temperature was 40°C. For 5FU a sodium acetate buffer (0.05 mol/l; pH 4.0) was used. MTX was eluted with a mixture of 72.5% sodium acetate buffer (0.05 mol/l; pH 4.0) and 27.5% methanol. The solvent flows were 1.0 ml/min. The HPLC system was equipped with an ultraviolet spectrophotometer (Pye Unicam LC-UV detector, Pye Unicam Ltd.). 5FU and MTX were analysed at 260 and 300 nm with retention times of 3.5 and 4.5 min, respectively. The selectivity of the method was verified by the analysis of blank and spiked samples and by taking full UV spectra.

Quantification (peak height) was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing 5FU or MTX dissolved in distilled water. Linearity was found until at least 15 mg/l (r >0.99). The limits of detection for 5FU and MTX were approximately 50 and 60 μg/l sample, respectively.

The recovery (±S.D.) of the wipe assay is 98±3% for 5FU and 81±25% for MTX (n=5). For the gloves it is 103±3% for 5FU and 61±20% for MTX (n=4).

GC analysis

Air samples, spot samples and gloves

1 ml Tris buffer (0.2 mol/l; pH 4.5) and 100 μl internal standard (IP (5 μg/ml distilled water) were added to 1 ml centrifuged sample. The samples were extracted twice with 5 ml ethyl acetate and the ethyl acetate layers were combined in conical tubes with screw caps. The extracts were dried under

nitrogen at 40°C and shaken with 50 µl ethyl acetate until the residue was totally dissolved 50 µl Trifluoroacetic anhydride were added and after shaking the tubes were closed for derivatization for 20 min at 70°C [41] The samples were cooled down to room temperature and dried again under nitrogen at 40°C 50 µl Toluene were added and after sonification during 3 min vials were filled with sample and stored at -20°C until analysis The recovery (±S.D.) of CP is 95±35% for the wipe assay and 58±17% for the gloves (n=6)

Urine samples

Instead of 1 ml centrifuged sample, 5 ml of urine sample were used The samples were extracted twice with 10 ml ethyl acetate. The ethyl acetate layers were combined in conical tubes with screw caps and washed successively with 5 ml of a 10% (wt/wt) sodium hydrogencarbonate solution and 5 ml of a hydrogen chloride solution (0 05 mol/l). The aqueous layers were discarded The organic phase was dried under nitrogen at 40°C The derivatization was performed in 100 µl ethyl acetate with 100 µl trifluoroacetic anhydride 500 µl Of a saturated sodium chloride solution were added to the samples After shaking, the samples were extracted twice with 5 ml *n*-hexane The *n*-hexane layers were combined in conical tubes and dried under nitrogen at 40°C. Finally, the dried samples were dissolved in 100 µl toluene

GC-MS conditions

GC-MS analysis was performed on a Varian Saturn GC-MS system which was controlled by a Compaq 386-20e personal computer (Varian, Houten, the Netherlands) The 'on column injection' mode was used (SPI Septum equipped temperature Programmable Injector) A 30 m DB-5 column (J & W Scientific, Folsom, California, USA) was used with 0 25 mm i.d and 0.25 µm film thickness The column was connected with a deactivated fused silica retention gap (Chrompack, Middelburg, the Netherlands) with a length of 2 5 m and 0 53 mm i d The initial injector temperature was 100°C After 1 min the temperature rose to 260°C (180°C/min) After 5 min at 260°C the temperature was lowered to the initial temperature by cooling with liquid carbon dioxide. The initial oven temperature was 100°C. After 1 min, the temperature rose to 280°C (15°C/min) where it remained constant for 5 min. Helium was used as carrier gas (column inlet pressure 100 kPa [14 psi]; column flow 0 8 ml/min) The interface temperature was 280°C and electron impact was used as ionization mode Identification was carried out by the combination of full scan spectra (250-399 atomic mass units) and retention time of CP Retention times of derivatized IP and CP were 10.4 and 11 1 min, respectively

Quantification was performed on the selected ion fragment 307, which was abstracted from the full scan spectra By using a high background mass we eliminated the unwanted matrix ions For quantification the peak area ratio of

CP/IP was calculated. Quantification of the trifluoroacetyl derivatives was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference (urine) samples containing CP dissolved in blank urine or distilled water (air samples, spot samples and gloves).
Frozen reference urine samples containing CP were analysed during a period of three months. No significant loss of CP was observed. The limit of detection was approximately 1 μg/l urine or distilled water.

Results

Air samples

Different volumes were sucked during stationary air sampling (23-92 m^3). Only on one filter (day F2, during preparation) MTX was found (0.3 $\mu g/m^3$). CP and 5FU were not detected.

Contamination in the laminar air-flow hood

The results of a wipe assay for the measurement of contamination with CP, 5FU and MTX in the laminar air-flow hood are presented in the Table 3. The amounts are divided by the area swept clean. Contamination with CP, 5FU and MTX was measured before and after preparation of the drugs and even after cleaning of the hood. Surprisingly, after cleaning, the drugs were measured at many spots whereas after preparation no contamination was detected at the same spots. For CP and for MTX, contamination was found after preparation. 5FU contamination was not found after preparation. Since many samples were positive before preparation and negative after preparation, the results suggest that CP, 5FU and MTX were removed from the surface very well during taking the first sample (before preparation). The overall degree of contamination per cm^2 was: MTX > 5FU > CP.

Contamination of the floor

In Table 4 an overview of the contamination on the floor of the preparation room is given. Before preparation of the drugs on day F1, 5FU was detected at all spots. Relatively high amounts were found in front of and beneath the laminar air-flow hood (11-16) and in the centre of the preparation room (17). The presence of 5FU at the entrance (outside) of the preparation room (18) was remarkable. Incidentally, low amounts of CP were measured in front of the laminar air-flow hood. MTX was not detected at all.

Table 3. Contamination with CP, 5FU and MTX of the laminar air-flow hood (ng/cm²) before and after preparation of the drugs and after cleaning of the laminar air-flow hood[a].

day	spot	CP			5FU			MTX		
		before preparation	after preparation	after cleaning	before preparation	after preparation	after cleaning	before preparation	after preparation	after cleaning
M1	1	-	-	7	-	-	-	-	-	-
	2	-	-	11	-	-	-	-	-	-
	4	-	-	3	-	-	-	-	-	-
	5	160	-	-	-	-	-	-	-	-
	6	-	2	-	-	-	-	-	-	-
F1	3	2	-	-	-	-	-	-	-	-
	5	-	12	-	-	-	-	-	-	-
	7[b]	12	3	1	10	-	-	200	-	-
	8[b]	15	10	14	53	-	27	633	243	293
M2	8	na	na	na	37	-	-	23	-	-
	9[c]	na	na	na	62	-	-	110	-	-
	10[c]	na	na	na	-	-	-	2	-	-
F2	6	na	na	na	-	-	-	-	-	90

[a] -: not detected; na: not analysed (no CP preparation)
[b] spot sample added on day F1
[c] spot sample added on day M2

Table 4. Contamination of the floor of the preparation room with CP, 5FU and MTX (μg/m²) before preparation of the drugs on day F1.

position	CP[a]	5FU	MTX[a]
11	-	122	-
12	-	182	-
13	-	88	-
14	-	148	-
15	2.6	236	-
16	1.4	162	-
17	-	126	-
18	-	48	-

[a] -: not detected

Glove contamination

The results of contamination of the gloves are shown in Table 5. It appeared that all gloves used, the left as well as the right ones, were contaminated with 5FU. MTX contamination was found to a lesser degree and CP was detected at only one pair (day F1; pair 2). Nearly all amounts of CP, 5FU and MTX measured were between 10 and 100 μg for each glove. No difference in 5FU contamination was found between the two workers. For each pair of gloves there was no systematic difference between the contamination on the left and the right-hand.

CP in urine

CP was not detected in any of the urine samples (n = 29). The detection limit was approximately 1 μg/l.

Table 5. Contamination with CP, 5FU and MTX of the gloves used during preparation of the drugs (µg/glove)[a].

day	glove	CP		5FU		MTX	
		left hand	right hand	left hand	right hand	left hand	right hand
M1	1	-	-	22	27	105	26
	2	-	-	666	58	-	-
	3	-	-	5	11	-	40
	4	-	-	135	135	-	-
F1	1	-	-	980	60	-	-
	2	17	20	66	58	-	-
	3	-	-	77	44	-	-
M2	1	na	na	27	27	156	-
	2	na	na	22	14	19	-
F2	1	na	na	19	22	-	-

[a] -: not detectable; na: not analysed (no CP preparation)

Discussion

From the contamination of the gloves, the laminar air-flow hood, the floor and one positive air sample, it can be concluded that release of drugs during preparation did happen.
On day M2 and day F2 preparation occurred while the viewing screen was not closed completely which might be responsible for the possitive air sample.
Contamination of the laminar air-flow hood was found for 5FU as well as for CP and MTX. Although much more 5FU was used than MTX, higher amounts of MTX were found in the laminar air-flow hood. Many spot samples were negative after preparation of the drugs but were positive before preparation. This suggests that the drugs were removed completely during the first sampling and, therefore, taking the wipe samples must be rather adequate. It is remarkable that spot samples were negative after preparation of the drugs but positive after cleaning of the hood. An explanation for these findings might be that during routine cleaning of the laminar air-flow hood with a gauze pad soaked in an alcohol solution these spots became contaminated from other surfaces which were cleaned with the gauze pad. In fact, this means that the laminar air-flow hood was not cleaned but that the contamination was spread out.

It was found that during the preparation of the drugs the gloves were contaminated with CP, 5FU and MTX. Whether this was due to contamination of the outside of the vials/ampoules or occurred while opening was not established.

It was striking that amounts of 5FU were found on the floor because a laminar air-flow hood was used and working procedures were performed according to special safety guidelines [38]. However, from observations of the working methods of the two workers it appeared that packing materials from disposables used for the preparation of the drugs were dropped on the floor in front of and beneath the laminar air-flow hood in order to maintain a good overview in the laminar air-flow hood. Because of the contamination of the gloves with 5FU, the packing materials could also be contaminated. The contamination might be transferred to the floor when the packing materials were dropped. Finally, it is possible that contamination was spread out unconsiously inside and outside the preparation room by the footwear of the workers or by cleaning the floor. The present study has demonstrated that when working procedures are not performed according to special safety guidelines, which do not allow to drop contaminated materials out of the laminar air-flow hood, contamination of the floor is possible. Contamination of the preparation room and spreading outside can possibly be reduced by dropping packing materials in a mini risk-container, which should be placed inside the laminar air-flow hood.

Several investigators have suggested that cytostatic drugs, like CP, are absorbed by inhalation and dermal penetration during occupational activities [33,36,37]. In two studies the excretion of unchanged CP was detected in urine of nurses involved in the preparation of cytostatic drugs [33,34]. After application of CP on the skin of volunteers (1 mg in methanol) low concentrations of CP were found in the urine [33]. The excretion rate was lower when compared to the excretion rate of CP in the urine from nurses involved in the preparation of CP, suggesting another route of absorption (inhalation). Also in rats after dermal application a lower excretion rate was observed compared to intratracheal instillation of CP (1 mg/kg bodyweight) [42].

In the present study, no uptake of CP was found by determination of CP in the urine. This might be due to the special safety guidelines and the security precautions taken [38]. However, in spite of the use of protective clothing, gloves and surgery masks, we recently found CP in the urine (0.7 μg/24 h) of an animal laboratory worker who was involved in the administration of CP to mice [43]. It is not clear to what extent the use of gloves by the hospital workers prevented the uptake of the drugs because only negative urine samples were found. The efficiency of protective gloves is discussed in literature [44-47]. Permeability of latex and polyvinyl chloride gloves to CP, 5FU, MTX and other cytostatic drugs was established. In general, the degree

of permeability appearred to be small for both types of gloves but lower permeability seemed to correspond with higher thickness. Contradictory results concerning the permeability to CP were found. The intralot variability in permeation suggested that exposure of personnel could be variable.
Another explanation for the absence of CP in the urine might be the complex metabolism of CP, which is found in patients but is unknown at low dose levels which might be expected after occupational exposure [48]. However, Hirst *et al.* and Evelo *et al.* did find CP in urine in similar circumstances [33,34]. Sensitive methods for the determination of CP metabolites and other cytostatic drugs or their metabolites are also necessary but unfortunately not available.
Although the uptake of CP by determination of CP in urine was not established, it is shown that the other methods applied in this study can be used for controlling the exposure to CP, 5FU and MTX.

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2.2

Occupational exposure to antineoplastic agents at several departments in a hospital. Environmental contamination and excretion of cyclophosphamide and iphosphamide in urine of exposed workers

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Summary

The occupational exposure to cyclophosphamide (CP), iphosphamide (IP), 5-fluorouracil (5FU), and methotrexate (MTX) of 25 pharmacy technicians and nurses from four departments of a hospital was investigated. Previously developed methods for the detection of exposure to some antineoplastic agents were validated. Exposure to CP, IP, 5FU, and MTX was measured by the analysis of these compounds in the environment (air samples and wipe samples from possible contaminated surfaces and objects). Contamination of the work environment was found not only on the working trays of the hoods and on the floors of the different rooms but also on other objects like tables, the sink unit, cleaned urinals and chamber pots, and drug vials and ampules used for preparation and packing of drugs. The gloves used during preparation of the drugs and during cleaning of the hoods were always contaminated. The uptake of CP or IP was determined by the analysis of both compounds in urine. CP or IP was detected in the urine of eight pharmacy technicians and nurses. The amounts ranged from <0.01 to 0.5 μg (median: 0.1 μg). CP and IP were found not only in the urine of pharmacy technicians and nurses actively handling these compounds ($n=2$) but also in the urine of pharmacy technicians and nurses not directly involved in the preparation and administration of these two drugs ($n=6$). CP and IP were excreted during different periods ranging from 1.40 to 24.15 h after the beginning of the working day, suggesting different times of exposure, different exposure routes, and/or interindividual differences in biotransformation and excretion

rate for these compounds. The urinary CP and IP determination method seems to be sensitive and suitable for monitoring the exposure to and measuring the uptake of these toxic compounds by pharmacy technicians and nurses during occupational activities.

Introduction

Chemotherapy with antineoplastic agents is often used in the treatment of cancer. In the Netherlands, the most frequently used antineoplastic agents for parenteral administration are cyclophosphamide (CP), iphosphamide (IP), 5-fluorouracil (5FU), and methotrexate (MTX) [8]. CP and IP are alkylating agents. These compounds are genotoxic after biotransformation [9]. Clinical studies show an increase in secundary tumors in patients treated with CP [2,5,6,10,12]. This is one of the reasons why the International Agency for Research on Cancer has classified CP as a human carcinogen [9]. There is limited evidence for the carcinogenicity of IP in mice and rats [9]. However, because of the structural resemblance of this compound to CP and the similarity with respect to biotransformation pathways and short-term genotoxicity results, IP may be considered a suspected carcinogen [9]. There are no indications for a carcinogenic potential of 5FU [9]. MTX is not classifiable with regard to its carcinogenicity; however, it is a human teratogen [9].

For genotoxic carcinogenic agents the absence of a no-adverse-effect level is supposed. Therefore exposure to these compounds should be avoided, and safety guidelines and protective measures like wearing masks, gloves, and gowns and using laminar air-flow safety hoods were introduced to protect the workers handling antineoplastic agents [1,16]. In order to monitor exposure levels and to measure possible uptake of these drugs it is necessary to use sensitive and compound-specific detection methods [4].

In this study we have validated some recently developed methods for the detection of contamination of the work environment with antineoplastic agents and for the determination of some of these drugs in urine of exposed workers [13]. We describe the occupational exposure of 25 hospital workers involved in handling antineoplastic agents at four departments in which CP, IP, 5FU, and MTX were prepared or administered. Exposure to these compounds was measured by the analysis of air samples and wipe samples from possibly contaminated surfaces and objects. The uptake of CP and IP was determined by the analysis of the unmetabolized form of one of both compounds in urine.

Materials and methods

Study design

Twenty-five female pharmacy technicians and nurses of four different departments [clinical pharmacy department, outpatient department (preparation), outpatient department (administration) and oncology department] participated in this study. Preparation of the drugs occurred in the clinical pharmacy department and in the preparation room of the outpatient department. In the administration rooms of the outpatient department and in the oncology department the drugs were not prepared but only administered. In the oncology department other nursing activities also took place, such as washing of the patients, urine collection, and cleaning of the beds and rooms of the patients.

The drugs were prepared in laminar air-flow safety hoods. The pharmacy technicians wore protective clothing, nurses caps, masks, and gloves. However, hardly any protective measures were taken by the nurses working in other departments.

It should be noted that many antineoplastic agents were prepared and administered but that in this study attention was given only to CP, IP, 5FU, and MTX. In all departments but one, CP, IP, 5FU, and MTX were prepared or administered. In the oncology department IP was administered but practically no CP or 5FU administration took place. Therefore in this department IP was determined in the urine of the nurses, whereas in all other departments CP was measured in urine of the pharmacy technicians and nurses.

Clinical pharmacy department

On two successive days, one air sample was taken during preparation of the drugs. Sampling occurred 1.5 m from the floor and approximately 0.5 m from the two pharmacy technicians who were working in the two hoods.

Contamination of the working tray of the two hoods (1.90 x 0.80 m) was measured on 4 days. Wipe samples were taken before and after the preparation of the drugs and after the cleaning of the hoods with an alcohol solution at the end of each working day.

Wipe samples were taken from the floor (0.70 x 0.70 m) of the preparation room on 2 days: on day 1 (at the beginning and end of the working day) and on day 4 (at the end of the working day). The samples were taken from the floor in front of each hood and in the center and at the entrance (inside and outside) of the preparation room. The total area of the preparation room was about $50 m^2$.

Contamination of the gloves used during preparation of the drugs and cleaning of the hoods was investigated. Twenty pairs of gloves were analyzed for contamination. Fourteen pairs were used for preparation of the drugs and six for cleaning of the hoods.

Before use, 9 CP vials, 20 5FU ampules, and 15 MTX vials were checked for contamination on the outside by taking wipe samples.
For each preparation, all necessary solutions and chemicals, needles, and syringes were put in a plastic box together with the preparation order. Contamination of nine of these boxes was measured by taking wipe samples from the inner surface.
Seventy-six packings of prepared cytostatic drugs (infusion bags, medication cassettes, and syringes) were checked for contamination by taking wipe samples on the outside.
Eleven pharmacy technicians were involved in the preparation of the drugs. Pharmacy technician 5 appeared twice in the research schedule. In addition to their work at the clinical pharmacy department, pharmacy technicians 4 and 9 were also working at the outpatient department (preparation). Pharmacy technicians 2, 5, 10, and 11 were not actively involved in the preparation of the drugs but collected all necessary items, including the drug for the preparation, and put them together with the preparation order in the boxes. During this investigation 18 g CP, 29 g 5FU, and 15 g MTX were prepared.

Outpatient department (preparation)
From the working tray of the hood (1.90 x 0.80 m) wipe samples were taken on 4 days before and after preparation of the drugs and after the cleaning of the hood with an alcoholic solution at the end of the working day.
Wipe samples were taken from the floor of the preparation room (0.70 x 0.70 m) on two days: on day 1 (at the beginning and end of the working day) and on day 4 (at the end of the working day). The wipe samples were taken in front of the hood and at the entrance (inside and outside) of the preparation room. The total area of the preparation room was about 12m^2.
Contamination of the gloves used for preparation of the drugs and for cleaning of the hood was analyzed. Eleven pairs of gloves were used.
The prepared drugs were packed ready to be transported and administrated in the administration rooms of the outpatient department. Nineteen packings (infusion bags, bottles, medication cassettes, and syringes) were checked for contamination by taking wipe samples from the outside.
Six pharmacy technicians were involved in the preparation of the drugs. Pharmacy technicians 4 and 9 were also working at the clinical pharmacy department. The worker coded number 14 was involved in the study as a pharmacy technician at the outpatient department (preparation) and as a nurse at the outpatient department (administration). During the investigation 2 g CP, 7 g 5FU, and 0.5 g MTX were prepared at this department.

Outpatient department (administration)
Five outpatient administration rooms (about 7-11 m^2) were used for the administration of the drugs. In each room wipe samples were taken twice on 4

days (at the beginning and end of the working day). The samples were taken from the floor (0.70 x 0.70 m) at the entrance (inside) of the administration rooms and from the area surrounding the beds and chairs used for the administration of the drugs.
Wipe samples were taken from the tables (1.20 x 0.70 m or 0.90 x 0.60 m) in the administration rooms to check for possible contamination with the drugs.
Four nurses were involved in the administration of the drugs.

Oncology department
In the so-called dirty room where urinals and chamber pots of the patients were collected and cleaned, samples were taken from the floor (0.70 x 0.70 m) on 4 days. Wipe samples were taken from the floor in front of the rinsing machine and at the entrance (inside) of the room. Wipe samples were also taken from the sink unit (0.80 x 0.50 m) where the urinals and chamber pots were collected.
The cleaning efficiency was checked for by taking wipe samples from the outside of 11 chamber pots and 9 urinals. Seven wipe samples were taken on two successive days from the floor (0.70 x 0.70 m) of four patient rooms.
Seven nurses were examined. Nurse 19 was involved twice.

Air sampling
Total airborne particulate matter was collected with a high volume sampler. The diameter of the cone was 20 mm and the suction flow was about 23.5 l/min. Cellulose filters (50 mm diameter, pore size 0.45 μm, Schleicher & Schuell, type RC 45, 's-Hertogenbosch, The Netherlands) were used. On day 1, 6.73 m^3 air was sampled, and on day 2, 5.55 m^3. The limits of detection for CP, 5FU, and MTX were 5, 30, and 300 ng/m^3, respectively.

Wipe sampling
Tissues (Kleenex professional wipes, 20 x 21 cm, Kimberly Clark Corp., Koblenz, Germany) were wetted with a sodium hydroxide solution (0.03 *M*) and the spots and objects were swept clean.
For the packings, boxes, chamber pots and urinals, two tissues and 5 ml of the above-mentioned solution were used. The floors, working trays of the hoods, the tables, and the sink unit were cleaned with four tissues and 10 ml solution. For the wipe samples taken from the floor, the tissues were not wetted but the solution was pipetted on the floor. The detection limits for the wipe samples were: floors and tables (CP = 0.02, 5FU = 0.1, MTX = 1 ng/cm^2), working trays of the hoods (CP = 0.01, 5FU = 0.04, MTX = 0.4 ng/cm^2), boxes and drug vials/ampules before and packings after preparation of the drugs (CP = 0.06, 5FU = 0.3, MTX = 3 μg).

Gloves

Gloves were only worn during preparation of the drugs. Two types were used (Perry White Latex surgical gloves and Perry Dermaguard surgical gloves, Smith & Nephew Medical, Massillon, Ohio, USA). After preparation of the drugs, the left and the right glove were collected together. The limits of detection for CP, 5FU, and MTX were 0.1, 0.7, and 6 μg/glove.

Sample preparation

After sampling, the filters, the tissues, and the gloves were put in a glass pot and a sodium hydroxide solution (0.03 M) was added (45 ml for the packings, boxes, chamber pots, and urinals, 90 ml for the floors, working trays of the hoods, tables, and sink unit). After sonification (90 min) and shaking (10 min), the extracts were centrifuged and the supernatant was analyzed by high-performance liquid chromatography (HPLC) and gas chromatography (GC). The gloves were only shaken (120 min).

Urine sampling

Total urine was collected in portions during the period of about 24 h starting from the beginning of the working day. The amounts excreted and the excretion periods were registered. The urine samples were stored at -20°C until sample preparation.

Chemicals

CP (Endoxan; purity >97%) and IP (Holoxan; purity >97%) were obtained from ASTA-Pharma (Bielefeld, Germany). 5FU (purity >99%) was purchased from Janssen Chimica (Beerse, Belgium) and MTX (purity >99.7%) was kindly donated by Pharmachemie (Haarlem, The Netherlands). Trifluoroacetic anhydride (purity >99%) was purchased from Johnson Mattey (Karlsruhe, Germany). All other chemicals were of the highest purity obtainable.

HPLC analysis (5FU and MTX)

Analysis of 5FU and MTX was performed on a reversed-phase HPLC system (PU LC3-XP). Twenty-microliter aliquots of the centrifuged extracts were injected onto a 150 x 4.6 mm Nucleosil RP 18 column with 5 μm particles (Machery-Nagel, Duren, Germany). The column temperature was 40°C. For 5FU a sodium acetate buffer (0.05 M; pH 4.0) was used. MTX was eluted with a mixture of 72.5% sodium acetate buffer (0.05 M; pH 4.0) and 27.5% methanol. The solvent flow rate was 1.0 ml/min in all cases. The apparatus was equipped with an ultraviolet spectrophotometer (PU LC-UV detector). 5FU

and MTX were analyzed at 260 and 300 nm with retention times of 3.5 and 4.5 min, respectively. The selectivity of the method was verified by the analysis of blank and spiked samples and by taking full UV spectra. Quantification (peak height) was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing 5FU or MTX dissolved in distilled water. The limits of detection for 5FU and MTX were approximately 7 and 60 μg/l sample, respectively.

GC analysis (CP and IP)

Air samples, wipe samples, and gloves
One milliliter TRIS buffer (0.2 M; pH 4.5) and 100 μl internal standard (5 mg IP/ml distilled water) were added to 1 ml centrifuged sample. After mixing, the samples were extracted twice with 5 ml ethylacetate and the ethyl acetate layers were combined in conical tubes with screw caps. The extracts were dried under nitrogen at 40°C and mixed with 50 μl ethyl acetate until the residue was totally dissolved. After addition of 50 μl trifluoroacetic anhydride and subsequent mixing, the tubes were closed for derivatization for 20 min at 70°C. The samples were cooled to room temperature and dried again under nitrogen at 40°C. Thereafter 50 μl toluene was added, and following sonification for 3 min, vials were filled with sample and stored at -20°C until analysis.

Urine samples
Five milliliters of urine was used instead of a 1 ml centrifuged sample. The samples were extracted twice with 10 ml ethyl acetate. The ethyl acetate layers were combined and washed successively with 5 ml of a 10% (w/w) sodium hydrogen carbonate solution and 5 ml of a hydrochloric acid solution (0.05 M). The aqueous layers were discarded. The organic phase was dried under nitrogen at 40°C. The derivatization was performed in 100 μl ethyl acetate with 100 μl trifluoroacetic anhydride. Thereafter 500 μl of a saturated sodium chloride solution was added to the samples. After mixing, the samples were extracted twice with 5 ml n-hexane. The n-hexane layers were combined in conical tubes and dried under nitrogen at 40°C. Finally the dried samples were dissolved in 100 μl toluene.
When IP was analyzed, CP was used as internal standard (5 mg CP/ml distilled water).

GC-MS conditions
GC-MS (gas chromatography-mass spectrometry) analysis was performed on a Varian Saturn GC-MS system which was controlled by a Compaq 386-20e personal computer. The on column injection mode was used (SPI: septum

equipped temperature programmable injector). A 30-m DB-5 column (J & W Scientific, Folsom, California, USA) was used with 0.25 mm internal diameter and 0.25 μm film thickness. The column was connected with a deactivated fused silica retention gap (Varian, Houten, The Netherlands) with a length of 5 m and an internal diameter of 0.53 mm. The initial injector temperature was 110°C. After 1 min the temperature was increased by 180°C/min to 280°C. After 8 min at 280°C the temperature was decreased to the initial temperature by cooling with liquid carbon dioxide. The initial oven temperature was 110°C. After 1 min, the temperature was increased by 15°C/min to 280°C, where it remained constant for 5 min. Helium was used as carrier gas (column inlet pressure 14 psi). The interface temperature was 280°C and electron impact was used as ionization mode. Identification was carried out by the combination of full scan spectra (250-399 amu) and retention times of CP and IP. Retention times of derivatized IP and CP were 10.4 and 11.1 min, respectively.
Quantification was performed on the selected ion fragment 307 which was abstracted from the full scan spectra. By using a high background mass we eliminated the unwanted matrix ions. For quantification the peak area ratio of CP/IP was calculated. Quantification of the trifluoroacetyl derivatives was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference (urine) samples containing CP and IP dissolved in blank urine or distilled water (air samples, wipe samples, and gloves).
Frozen reference urine samples containing CP or IP were analyzed over a period of 3 months. No significant loss of CP was observed. The limit of detection for both compounds is about 0.1 μg/l urine or distilled water.

Results

Clinical pharmacy department

In the two air samples taken during preparation of the drugs no CP, 5FU, or MTX was detected.
Contamination of the working tray with 5FU was detected once before preparation in both hood 1 and hood 2 (Table 1). CP was measured once after preparation in hood 2 (0.1 ng/cm^2; data not shown). No MTX contamination was observed.
Contamination of the floor with 5FU was found at all locations on at least one of the two days, except in front of hood 2, where no 5FU was detected (Table 2). CP and MTX were not detected in any of the wipe samples taken from the floor.
The gloves appeared to be contaminated during preparation of CP, 5FU, and MTX (Table 3). The gloves were found to be contaminated with 5FU and MTX not only during preparation of these two drugs but also during preparation of other drugs. CP contamination was found once during preparation of other

antineoplastic agents. No significant correlation was found between the contamination with 5FU and the amounts (total mass or number of ampules) of 5FU prepared. Many of the gloves used for cleaning the hoods were contaminated with CP, 5FU and/or MTX (Table 4). Contamination with 5FU was found most frequently.

Some of the packings of the drugs were contaminated on the outside. One vial of CP was contaminated with 0.06 μg CP and one vial of MTX had 15 μg MTX on the outer surface.

No contamination was observed on the boxes in use for the collection of the solutions and chemicals necessary for preparation of the drugs.

On the outside of the packings of four prepared products (two 5FU cassettes and one 5FU and one IP infusion bag) 5FU was found (1.4, 3.1, 4.0, and 6.2 μg, respectively). CP was detected once on a CP infusion bag (2.1 μg).

Table 1. Contamination of the working tray of the hoods with 5FU in two departments (ng/cm²).

day	sampling time	clinical pharmacy department		outpatient department (preparation)
		hood 1	hood 2	
1	bp[a]	nd[d]	nd	1.4
	ap[b]	nd	nd	1.8
	ac[c]	nd	nd	0.6
2	bp	0.5	0.2	0.3
	ap	nd	nd	0.6
	ac	nd	nd	nd
3	bp	nd	nd	0.2
	ap	nd	nd	0.5
	ac	nd	nd	nd
4	bp	nd	nd	nd
	ap	nd	nd	nd
	ac	nd	nd	nd

[a] bp: before preparation
[b] ap: after preparation
[c] ac: after cleaning
[d] nd: not detectable (<0.04 ng/cm²)

Outpatient department (preparation)
Before as well as after preparation, 5FU contamination of the working tray of the hood was detected on the first 3 days (Table 1). Contamination with MTX was not found. CP was only detected on day 2 after preparation (12 ng/cm^2). Contamination of the floor with 5FU was found on both days (Table 2). CP and MTX were not detected in any of the spot samples.
During preparation of the drugs and during cleaning of the hood 11 pairs of gloves were used (seven and four pairs, respectively). On the gloves worn during preparation of the drugs only contamination with 5FU was found (Table 3). 5FU was also present on gloves not used for the preparation of 5FU. A significant correlation was found between the contamination of the gloves with 5FU and the amount of 5FU prepared with them (Spearman's coefficient of rank correlation: $r_s = 0.78$, $P < 0.05$). Many of the gloves used for cleaning the hood were contaminated with 5FU and MTX but not with CP (Table 4).
The outside of the packings of the prepared drugs was not contaminated with CP, 5FU, or MTX.

Table 2. Contamination of the floor with 5FU (ng/cm^2).

department	day 1		day 4
	beginning of working day	end of working day	end of working day
clinical pharmacy department			
in front of hood 1	0.2	nd[a]	nd
in front of hood 2	nd	nd	nd
center of the room	3.1	1.1	nd
inside the room	0.8	2.1	2.9
outside the room	5.5	3.3	10.7
outpatient clinical department (preparation)			
in front of the hood	1.2	1.1	nd
inside the room	2.6	3.1	2.9
outside the room	2.1	0.5	2.5

[a] nd: not detectable (< 0.1 ng/cm^2)

Outpatient department (administration)
Contamination of the floors of the administration rooms was detected during

all 4 days of the investigation (at the beginning and end of the working day). The amount of 5FU (n = 10) decreased from a median value of 1.8 ng/cm^2 on day 1 (beginning of the working day) to a median value of 0.7 ng/cm^2 on day 4 (end of the working day) (data not shown). CP was only found on the floor at the beginning of the working days and mostly only around the beds and chairs (n = 5) used for the administration (<0.02-4.5 μg/cm^2) (data not shown). Contamination with MTX was not found.
On the tables in the administration rooms, 5FU contamination was found once before administration (day 1: 22 μg) and twice after administration (day 1: 6.5 μg; day 3: 4.9 μg). CP contamination was detected at the beginning of working day 1 on one table (4.5 μg) and on day 2 on three tables (0.2, 0.3, and 0.7 μg).

Oncology department
On the floor of the "dirty room", CP was detected once in front of the rinsing machine (0.9 ng/cm^2). MTX contamination was not observed. MTX was measured in three wipe samples taken from the floor of two patient rooms (5.5, 5.5, and 5.9 ng/cm^2). CP contamination of the floors was not observed. CP was detected on one urinal (8.3 μg). MTX was not found on the urinals or chamber pots.

Table 3. Contamination of the gloves used during preparation of the drugs[a] (μg/glove).

department	CP+[b]	CP-[b]	5FU+	5FU-	MTX+	MTX-
clinical pharmacy department						
pairs used	2	12	6	8	9	5
pairs contaminated	0	1	6	8	2	1
range (μg)	-	<0.1-21	23-62	19-87	<6-49	<6-31
median (μg)	-	<0.1	59	36	<6	<6
outpatient department (preparation)						
pairs used	2	5	4	3	5	2
pairs contaminated	0	0	3	2	0	0
range (μg)	-	-	<0.7-140	<0.7-30	-	-
median (μg)	-	-	54	18	-	-

[a] limits of detection: CP = 0.1, 5FU = 0.7, and MTX = 6 μg/glove
[b] + : gloves used for the preparation of the indicated drug
- : gloves not used for the preparation of the indicated drug

Table 4. Contamination of the gloves used for cleaning the hoods[a] (μg/glove).

department	CP	5FU	MTX
clinical pharmacy department			
pairs used	6	6	6
pairs contaminated	1	6	2
range (μg)	<0.1-11	25-59	<6-49
median (μg)	<0.1	49	<6
outpatient department (preparation)			
pairs used	4	4	4
pairs contaminated	0	3	2
range (μg)	-	<0.7-18	<6-40
median (μg)	-	10	18

[a] limits of detection: CP = 0.1, 5FU = 0.7, and MTX = 6 μg/glove

CP and IP in urine

The urine analysis data for CP and IP obtained from the urine samples of the pharmacy technicians and nurses are given in Table 5. CP and IP were detected in the urine samples of 8 of 25 pharmacy technicians and nurses from all four departments. The amounts excreted ranged from 0.01 to 0.5 μg (median: 0.1 μg). In urine of two of these workers CP or IP was identified but the amounts were too low for quantification (signal to noise ratio < 3). In urine of 17 other workers neither CP nor IP was detected (< 0.1 μg/l). CP and IP were not only measured in urine of pharmacy technicians and nurses actively handing these compounds (n = 2) but also in urine of pharmacy technicians and nurses who were not directly involved in the preparation and administration of these drugs (n = 6).

Since total 24-h urine was collected and the individual portions were analyzed, the excretion periods can be indicated with the time at zero at the beginning of the working day. In fact this is the time at which the exposure also might have started. The data in table 5 show different "excretion periods" for all pharmacy technicians and nurses. CP and IP excretion was complete within 24 h after the beginning of the working day. In all pharmacy technicians and nurses CP was measured in only one urine portion except for nurse 14. She excreted CP in two successive fractions. Nurse 19 took part in this study on two successive days. On both days IP was measured in her urine. On the first day, the "excretion period" was 5-8.5 h after the beginning of the working day. On the second day the "excretion period" was 18.25-24 h.

Table 5. Urinary CP or IP excretion[a].

department	nurse/ pharmacy technician	CP or IP[b] preparation/ administration (mg)	CP or IP in urine	
			amount (μg)	excretion period[c] (h.min)
clinical pharmacy	1	4200	0.1	12.45-13.40
department	2	-	0.3	22.45-24.15
	3	-	0.5	12.00-13.30
	4[d]	1500	nd[e]	-
	5	4200	nd	-
	6	5700	nd	-
	7	2600	nd	-
	8	4750	nd	-
	5,9-11[d]	-	nd	-
outpatient department	12	-	0.07	2.10-7.00
(preparation)	13	-	< 0.01[f]	1.40-3.45
	4[d]	600	nd	-
	9[d]	1750	nd	-
	14[g],15	-	nd	-
outpatient department	14[g,h]	-	0.07	8.20-10.30
(administration)			0.04	10.30-19.00
	16	2300	0.1	10.25-11.00
	17,18	-	nd	-
oncology department[a]	19	-	< 0.01[f]	5.00-8.30
	19	-	0.4	18.15-24.00
	20	4800	nd	-
	21	1000	nd	-
	22-25	-	nd	-

[a] at all departments except for the oncology department urine samples were analysed for the presence of CP
at the oncology department the urine samples were analysed for the presence of IP

[b] -, neither CP nor IP preparation or administration

[c] "excretion period" is defined as follows: starting at the time of the previous urine production and ending at the time of the production of this particular urine sample, with the time at zero at the beginning of the working day

[d] pharmacy technician 4 and 9 were working at both departments

[e] nd, not detectable (<0.1 μg/l urine)

[f] positively identified but not quantified (signal/noise ratio <3)

[g] pharmacy technician/nurse 14 was working at both departments

[h] CP was detected in two successive urine portions

No significant correlations were observed between the amounts of CP and IP excreted in urine and the amounts of CP and IP prepared or administered. Also no correlation was observed between the amounts of CP and IP excreted in urine and smoking habits, the use of alcohol, or the use of hormonal contraceptives.

Discussion

In the biomonitoring studies directed at the detection of occupational exposure to antineoplastic agents, hardly any data have until now been published about environmental contamination due to spillage during working procedures [15]. In two studies in hospital departments, air-contamination with cytostatic drugs was found, which suggested that inhalation was a potential absorption route [11,17].

Recently we published the results of an investigation in a pharmacy department of a hospital in which the occupational hygiene of preparing cytostatic drugs was studied [13]. Environmental contamination with CP, 5FU, and MTX was observed but no CP was detected in urine of the pharmacy technicians preparing these drugs. In the present investigation not only was environmental contamination found, but CP and IP were detected in the urine of pharmacy technicians and nurses whether or not they were directly involved in the preparation and administration of these drugs.

In the present study, the preparation of the drugs occurred in the clinical pharmacy department and in the preparation room of the outpatient department. The working conditions and the operating procedures in both departments were essentially the same. Protective clothing, hair-nets, masks, and gloves were worn and the drugs were prepared in laminar air-flow safety hoods. Therefore, it is not suprising that the findings in these departments show comparable results. The results of our investigation demonstrated that handling of CP, 5FU, and MTX resulted in contamination of the environment.

In the wipe samples taken from the floor only 5FU was found. It was remarkable that 5FU was also measured outside the preparation room. During preparation, contamination of the working trays of the hoods with CP and 5FU was found occasionally in the clinical pharmacy department. Frequent contamination with 5FU of the working tray of the hood in the preparation room of the outpatient department was observed and the amounts were higher than in the clinical pharmacy department. Not only preparation of the drugs but also cleaning of the hoods resulted in contamination of the gloves. Contact with these gloves may result in further contamination such as was found on the packings of the prepared products. The boxes, however, were not contaminated. Drugs on the outside of the vials and ampules may also be a source of contamination.

Administration of the drugs took place in the administration rooms of the outpatient department and in the patient room of the oncology department. Hardly any safety measures or precautions were taken by the nurses. It should be emphasized that in the outpatient department only administration of the drugs took place whereas in the oncology department also other activities were also carried out (washing of the patients, urine collection, and cleaning of the beds and rooms of the patients). This may have resulted in exposure to the drugs otherwise than during administration. In both departments environmental contamination was found. Not only on the floors of the administration rooms and the rooms of the patients, but also on the tables in the administration rooms, small quantities of cytostatic drugs were found. It is surprising that the floors of the administration rooms were contaminated with CP at the beginning of the working day although no CP was detected the day at the end of the previous working day. It should be mentioned that in the meantime the rooms had been cleaned. This finding suggests that the administration rooms were not cleaned efficiently and that the contamination may have been spread. Since the administration rooms of the outpatient department and the rooms of the patients in the oncology department were also accessible to people other than nurses (family and friends of the patients), these rooms may also be a source of exposure for these persons.

In the so-called dirty room urinals and chamber pots were collected on the sink unit for registration. Thereafter, they were cleaned in the rinsing machine. These urinals and chamber pots may be potential sources of cytostatic drugs which could be responsible for the contamination of the sink unit and the floor in front of the rinsing machine. Although the chamber pots and urinals were cleaned in the rinsingmachine, the outside of some was still contaminated.

CP and IP were detected in urine of eight pharmacy technicians and nurses working in all departments under investigation. It is remarkable that CP and IP were also measured in urine of pharmacy technicians and nurses not directly involved in the preparation or administration of these two drugs. This suggests exposure sources other than preparation or administration, e.g., contact with contaminated surfaces. In general, biological monitoring is applied in addition to environmental monitoring to procure maximum information about the exposure of the persons involved and to have possibilities to study the role of the various exposure routes. Measurements in urine do not per se reflect measurements in the environment. In many cases where dermal exposure may occur, like in this study, it is impossible to determine the total environmental exposure. For that purpose one would have to measure everything the workers touch or breathe.

We were interested in a relation between the working period and the time of excretion of CP/IP. Therefore we have indicated the period of excretion of CP/IP in relation to the time from the beginning of the working day (Table 5). In all workers the excretion intervals started at different times. This might be

explained by different times of exposure, different exposure routes, and interindividual differences in biotransformation and/or excretion of CP and IP. Our results show different periods from the beginning of the working day until the time of urinary CP excretion, ranging from 2 to 24 h. In the case of nurse 19 two rather different periods were found, suggesting different times or routes of absorption of IP since differences in biotransformation and excretion are less likely.

For antineoplastic agents, dermal uptake and inhalation are suggested as routes of exposure. In two studies the excretion of unchanged CP was detected in urine of nurses involved in the preparation of CP [3,7]. In the study by Evelo et al. CP was measured in total 24-h urine samples [3]. In a study by Hirst et al. the times of handling CP and the periods of the collection of the urine samples were indicated with respect to the end of the working day [7]. After application of 1 mg CP on the skin of volunteers, low concentrations of CP were found in urine. The excretion rates were lower than the excretion rates of CP in urine from nurses involved in the preparation of CP, suggesting another route of absorption (inhalation). It was not possible to establish whether CP was excreted over a longer period, as might be caused by dermal uptake, since urine samples were collected from the nurses only at the end of the working day. Therefore the suggestion that CP was absorbed by inhalation could be premature. Evidence for a different excretion rate after dermal application in comparison to exposure via inhalation was obtained in a toxicokinetic study using rats. In this study lower urinary excretion rates were observed after dermal application than after intratracheal instillation of CP [14].

Conclusion

Environmental contamination was found in all four departments, not only on the floors of the different rooms and on the working trays of the hoods but also on other objects (tables, sink unit, cleaned urinals and chamber pots, the packings of the prepared and unprepared drugs). In urine of pharmacy technicians as well as nurses, CP or IP was detected. Although safety precautions were taken during preparation of the drugs in the clinical pharmacy department and in the preparation room of the outpatient department, CP was measured in urine of some pharmacy technicians working in these departments. How this might have happened remains unclear and will be the subject of further study. It was also shown that preparing or administering CP or IP was not the only determining factor since CP or IP was detected in urine of pharmacy technicians and nurses not directly involved in these tasks. The urinary CP/IP determination method seems suitable for controlling the exposure to and measuring the uptake of these toxic compounds.

Acknowledgement

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2.3

Determination of cyclophosphamide in urine by gas chromatography-mass spectrometry

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Abstract

A sensitive gas chromatographic method for the determination of cyclophosphamide (CP) in urine is presented. After liquid-liquid extraction with diethyl ether and derivatization with trifluoroacetic anhydride, CP was identified and quantified with mass spectrometry. The method is suitable for the determination of CP at concentrations of more than 0.25 ng/ml, which enables the uptake of CP during occupational activities, such as the preparation and administration of antineoplastic agents in hospitals, to be measured. Simple preparation makes the method appropriate for routine analysis.

Introduction

Antineoplastic agents are widely used in cancer chemotherapy. When handling antineoplastic agents, pharmacy technicians and nursing personnel may face certain health risks. There is sufficient evidence that several antineoplastic agents are carcinogenic to humans, because secondary cancers are found in cancer patients treated with these drugs, and carcinogenic effects have been observed in non-cancer patients treated with these therapeutics for other purposes [1]. Special guidelines and extensive safety precautions were introduced in the Netherlands about ten years ago, to protect workers from contact with these toxic compounds [2]. Recently, these guidelines were amended [3].
In order to measure possible uptake of these compounds by workers, it is neccessary to use sensitive detection methods. Non-specific detection

methods, such as urinary mutagenicity, analysis of chromosome aberrations and sister chromatid exchanges in blood lymphocytes, have been used for the determination of occupational exposure to antineoplastic agents [4,5]. These methods are suitable in cases when hospital workers had a minimum or a lack of protective measures against possible exposure to cytostatic drugs. Sensitive methods have been scarcely developed.
We have studied the amounts of drugs used in the Netherlands over the years 1986 to 1988 [6]. We found that CP was prepared and administered in Dutch hospitals most frequently. Therefore and because of CP's carcinogenic potency, we chose to concentrate of this drug.
A literature review showed that the most suitable method for the detection of CP was derivatization with trifluoroacetic anhydride followed by gas chromatography (GC) with nitrogen-phosphorus, electron-capture, or mass-selective detection [7-10]. Also, a method without derivatization using GC with electron-capture detection has been published [11]. Unfortunately, some of these methods are not sensitive enough for the detection of exposure to the low levels of CP in urine expected during occupational activities under the regimen of the present Dutch guidelines [7,8], and others are time-consuming and not suitable for routine analysis [9,10].
This paper describes a GC method with liquid-liquid extraction, derivatization with trifluoroacetic anhydride and mass spectrometric (MS) detection. The method is sensitive and simple in practice, which makes it suitable for routine analysis.

Experimental

Materials and reagents

CP (Endoxan) and iphosphamide (IP) (Holoxan) were purchased from ASTA-Pharma (Bielefeld, Germany). Trifluoroacetic anhydride was obtained from Johnson Mattey (Karlsruhe, Germany). All other chemicals were of the highest purity obtainable.

Sample preparation

A 0.5-ml volume of 1M Tris buffer (pH 8.0) and 100 μl of the internal standard IP (5 μg/ml in distilled water) were added to a 5-ml urine sample. After mixing,

the samples were extracted twice with 20 ml of diethyl ether[1], a and the ether layers were combined and evaporated under nitrogen at 30°C until a residue of ca. 2 ml was obtained. The solution was transferred to conical tubes with screw caps and further evaporated to dryness. Then 100 µl of ethyl acetate were added, and the solution was mixed until the residue was totally dissolved. Trifluoroacetic anhydride (100 µl) was added and, after mixing, the tubes were closed for derivatization for 20 min at 70°C. The samples were cooled to room temperature and evaporated to dryness under nitrogen at 40°C. The dried samples were dissolved in 100 µl of toluene, mixed and sonificated for 5-10 min. Vials were filled with sample and stored at -20°C until analysis.

Calibration

Calibration curves were constructed from the analysis of standard urine samples, which were freshly prepared by adding CP to blank urine. The CP concentrations of the standard urine samples were 0, 2, 10, 20, 50, and 100 ng/ml urine.

A control urine sample was obtained by adding CP to pooled blank urine (11.7 ng/ml). From this control urine sample, aliquots of ca. 10 ml were stored at -20°C. These aliquots were analysed in duplicate on every occasion when urine samples were assayed. The results from these determinations were used to calculate the inter- and intra-assay precision.

Gas chromatographic-mass spectrometric analysis

The samples were analysed on a Varian Saturn GC-MS ion-trap system with a Varian 8100 autosampler, which was controlled by a Compaq 386-20e personal computer (software version B). The on-column injection mode was used (SPI: septum equipped temperature programmable injector). Separation was carried out on a fused-silica capillary column (DB-5, 30 m x 0.25 mm I.D., film thickness 0.25 µm). The column was connected to a deactivated fused-silica retention gap (Varian, 5 m x 0.53 mm I.D.). The initial injector temperature was 110°C. After 1 min, the temperature was increased by 180°C/min to 280°C. After 8 min at 280°C, the temperature was decreased to the initial temperature by cooling with liquid carbon dioxide. The initial oven temperature was 110°C. After 1 min, the temperature was increased by 15°C/min to 280°C, where it remained constant for 5 min. Helium was used

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[1] Previously we used ethyl acetate with two clean-up steps. But greater amounts of solvents should be used and, after derivatization with trifluoroacetic anhydride, an additional extraction with n-hexane is needed to reach the same detection limit.

as carrier gas (column inlet pressure 96 kPa). The interface temperature was 280°C. Electron impact (EI) was used as ionization mode. Identification was carried out by the combination of full-scan spectra (m/z = 250-399) and retention times of CP and IP. Retention times of derivatized IP and CP were 9.40 and 10.20 min, respectively. Quantification of the N-trifluoroacetyl derivatives was performed on the selected-ion fragment m/z 307, which was abstracted from the full-scan spectra. By using a high background mass, the unwanted matrix ions were eliminated. For quantification, the CP/IP peak area ratio was calculated.

Results and discussion

Mass spectra of N-trifluoroacetylated CP and IP are shown in Fig. 1. High relative intensity was found for m/z 307 ($M^+ + 1\text{-}CH_3Cl$) for both compounds, because the molecular fragment m/z 356 was not stable under EI conditions [7,8]. Fig. 2 shows total and reconstructed (m/z 307) ion chromatograms of a blank urine sample spiked with CP and IP (A), a blank urine sample (B), and a urine sample from a CP-exposed pharmacy technician involved in the preparation of CP (C). CP and IP were completely separated, and no interference with other compounds was observed. When the selectivity was increased by the use of the reconstructed ion fragment (m/z 307) a higher sensitivity was obtained (Fig. 2A). The use of chemical ionization instead of EI did not result in a higher sensitivity.

The clean-up and derivatization procedure sounds similar to already published methods. The difference is the use of an ion-trap mass spectrometer instead of a quadrupole device with selected-ion monitoring (SIM). In previous unpublished work we found that, in this particular case for the determination of CP in urine, the ion-trap spectrometer is more sensitive than a quadrupole device with SIM.

Thirty frozen control urine samples containing 11.7 ng/ml CP were analysed in duplicate during a period of three months. No significant loss of CP was observed, which means that the medium-term stability is good. The mean CP concentration of these analyses was 9.9 ng/ml. The coefficient of variation (C.V.), as a measure of the inter-assay precision, was 18%. The intra-assay precision was 5.1%. The calibration curves were linear, with a coefficient of correlation of at least 0.99. The limit of detection was ca. 0.25 ng/ml urine (12.5 pg on column) with a signal-to-noise ratio of more than 3.

According to the International Agency for Research on Cancer, CP is a human genotoxic carcinogen (group 1). This means that exposure to this compounds should be avoided because any detectable level is considered to be a hazard.

This supports the need for a sensitive method for the determination of CP in urine.

The method presented in this study is sensitive and suitable for the detection of CP in urine at the low ng/ml level. This is the level we have found in workers occupationally exposed to this carcinogenic drug [12-14].

Fig. 1. Mass spectral fragmentation of N-trifluoroacetylated CP and IP (internal standard).

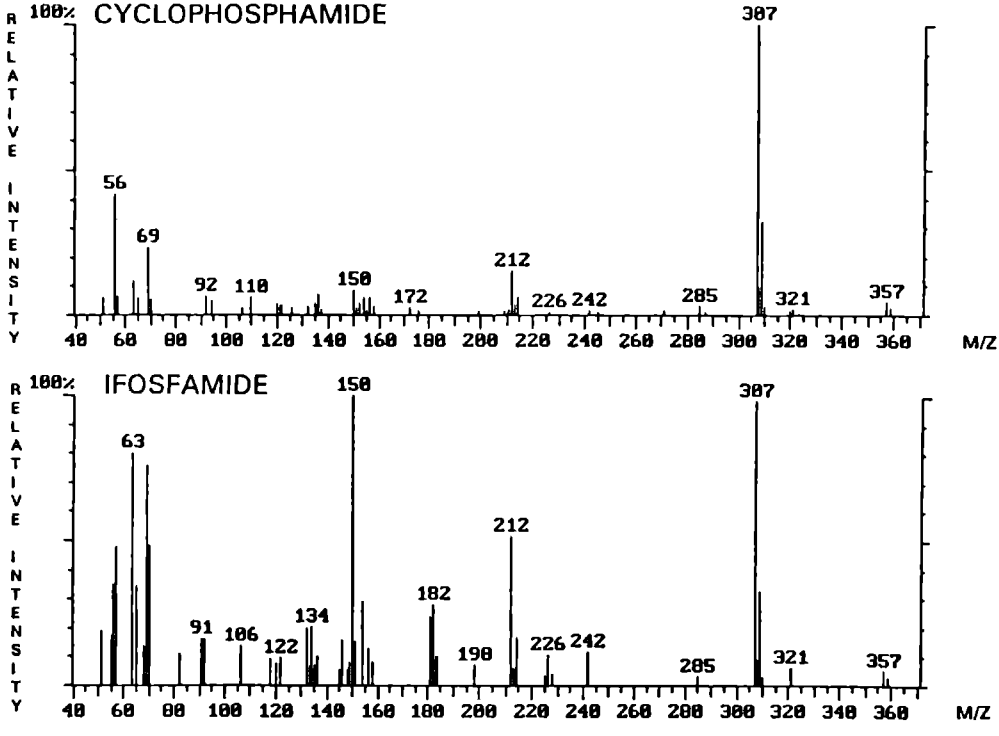
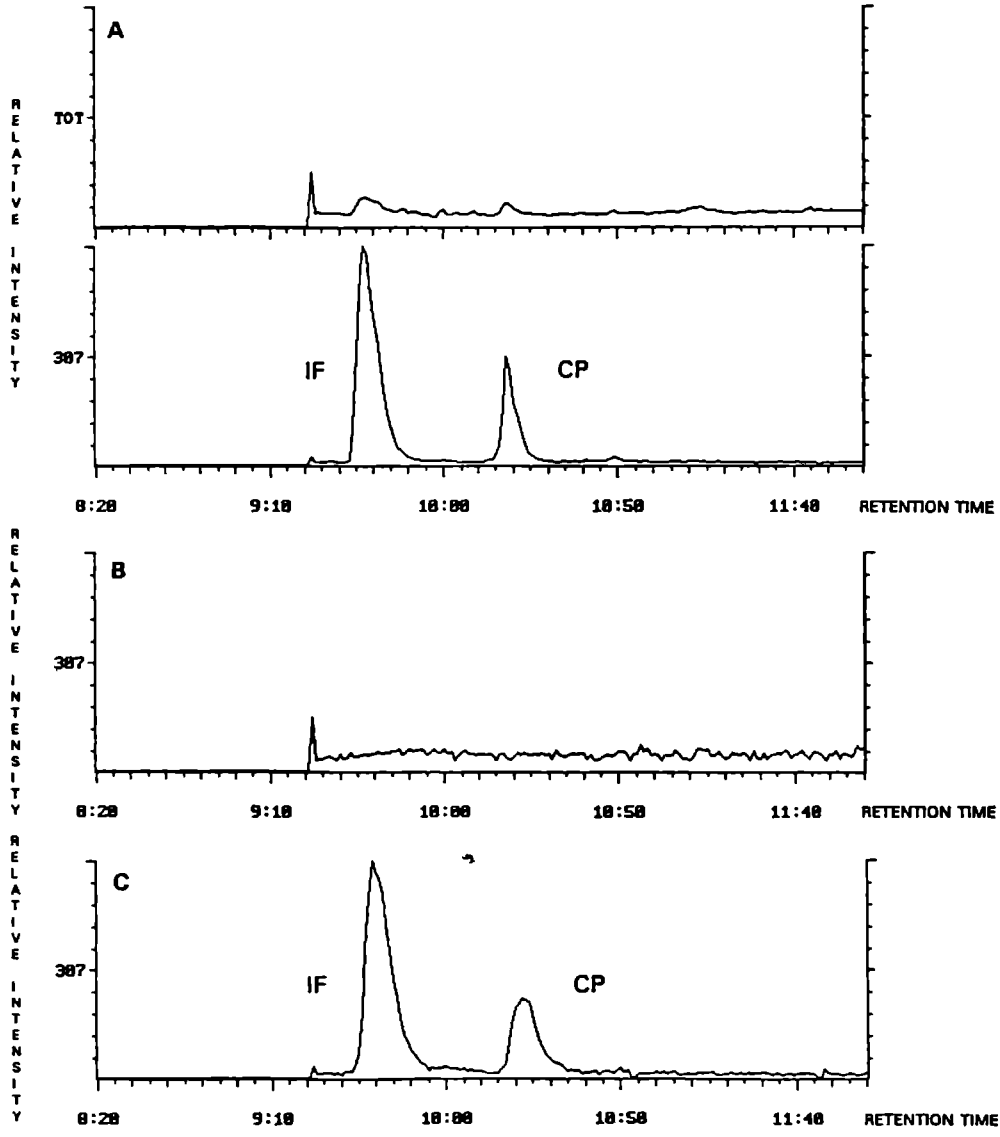


Fig. 2. Total and reconstructed (m/z 307) ion chromatograms. Filament and multiplier were switched on at 9:20 min. (A) A blank urine sample spiked with 10 ng/ml CP and 100 ng/ml IP (internal standard). (B) A blank urine sample. (C) A urine sample of a pharmacy technician involved in the preparation of antineoplastic agents, including CP. The concentrations of CP and IP were 18 and 100 ng/ml.



Appendix

Introduction

EI ionisation with the reconstructed (m/z = 307) ion chromatogram is specific for CP and IP. However, specificity is increased by using MSMS. Thereto, the parent ion mass m/z = 307 is stored in the ion-trap and further split in a so-called daughter ion with m/z = 212 which is very specific for CP and IP. This new application has resulted in some changes in the method which are briefly discussed below.

Gas chromatographic-mass spectrometric analysis

The samples were analysed on a Varian Saturn 4D GC/MS/MS ion-trap system with a Varian 8200 autosampler, which was controlled by a Compaq Prolinea 4/50 personal computer (software version 5.2). The initial injector temperature was 110°C. After 1 min, the temperature was increased by 180°C/min to 280°C. After 5 min at 280°C, the temperature was decreased to the initial temperature by cooling with compressed air. The initial oven temperature was 110°C. After 1 min, the temperature was increased by 15°C/min to 290°C, where it remained constant for 7 min. The interface temperature was 290°C. Daughter-ion m/z = 212 was produced at an excitation amplitude of 43V (IP) and 46V (CP) (nonresonant) and an excitation RF of 500 dac. By using a high background mass (m/z = 95), the unwanted matrix ions were eliminated. Consequently background noise is further reduced and the sensitivity is improved. Quantification was performed on the daughter ion mass m/z = 212.

Results and discussion

MSMS mass spectra of the parent ion mass m/z = 307 of N-trifluoroacetylated CP and IP are shown in Fig. 3. High relative intensity was found for daughter ion mass m/z = 212 for both compounds. Fig. 4 shows the reconstructed (m/z = 307) ion chromatogram of a blank urine sample spiked with CP and IP (1 μg/l urine) obtained by EI and a chromatogram of the daughter ion mass m/z = 212 of the same sample obtained by MSMS. It is shown that the daughter ion mass m/z = 212 obtained by MSMS further increased the specificity and the selectivity of the method. Retention times of derivatized IP and CP were 8.6 and 9.3 min, respectively. CP and IP were completely seperated, and no interference with other compounds was observed. The limit of detection is 0.1 ng/ml urine.

Fig. 3. MSMS mass spectra of the parent ion mass m/z = 307 N-trifluoroacetylated CP and IP (internal standard).

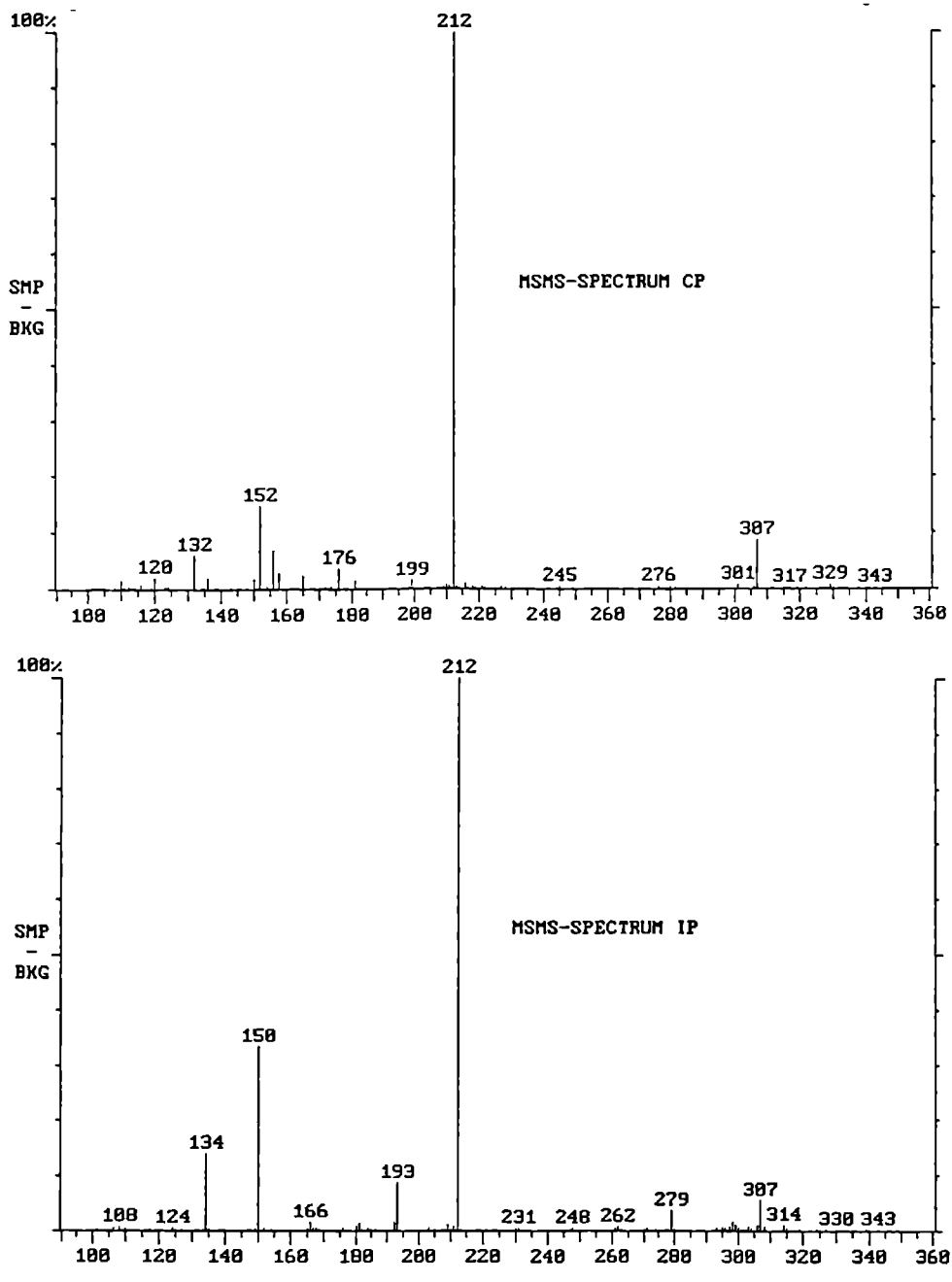
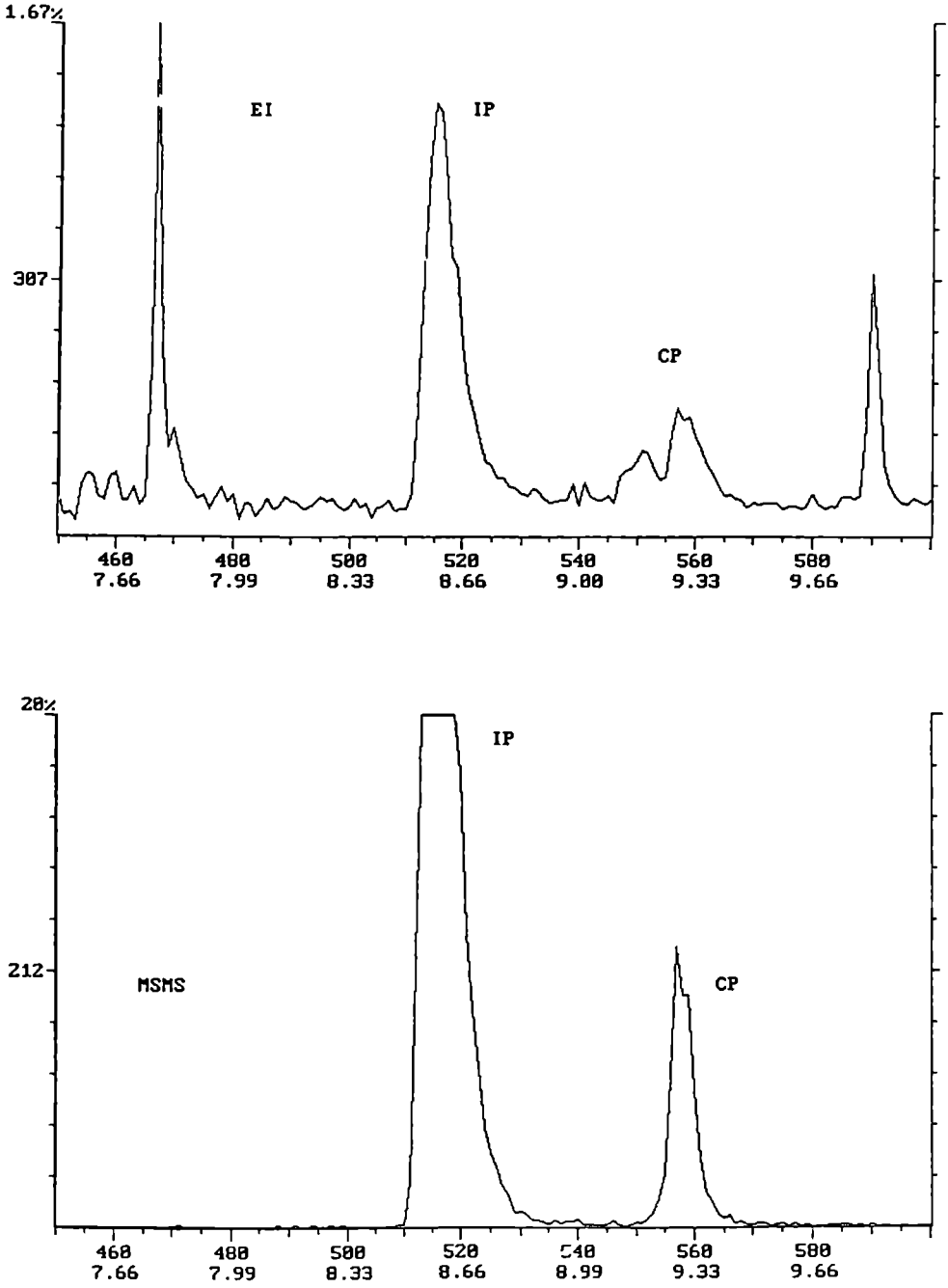


Fig. 4. Reconstructed (m/z 307) ion chromatogram of a blank urine sample spiked with CP and IP (1 µg/l urine) and a chromatogram of the daughter ion mass m/z = 212 of the same sample obtained by MSMS.



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2.4

Occupational exposure of animal keepers to cyclophosphamide

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Abstract

Little is known about the exposure of animal keepers to toxic agents during the administration of such chemicals to laboratory animals. In this study, we have investigated the environmental contamination with cyclophosphamide (CP) in an animal laboratory where mice were housed and injected with this compound. Also the contamination of gloves, sleeve protectors, and masks used for personal protection was studied. The uptake of CP by the animal keepers was determined by the analysis of unmetabolized CP in urine. For the estimation of CP in the air, air samples were taken and filters of the air-circulation system were analyzed. On the filters, amounts of CP were detected corresponding with <0.1-1.0 μg/day. Environmental contamination was also measured by analysis of wipe samples taken from different spots (objects and surfaces). The presence of CP was not only observed in the room where the mice were housed and treated with CP but also in adjacent rooms (<0.02-44 ng/cm^2). The gloves used during the injection of CP were always contaminated (2-199 μg/pair). No penetration of the gloves was established. The sleeve protectors were incidentally contaminated (<0.3-10 μg) and on the masks no CP was found (<0.2 μg). Eighty seven urine samples from four animal keepers were analyzed for unmetabolized CP. In one sample CP was detected (0.7 μg). The results show that in this particular study animal keepers are exposed to CP during their work.

Introduction

In the Netherlands, about 2000 persons are potentially involved in the administration of chemicals to laboratory animals for medical, toxicological, or other purposes. Although the number of persons is not very large, they may be exposed to many different chemical substances. Appropriate methods for controlling their exposure to toxic agents are limited. Little is known about their exposure to the many different toxic chemicals with which they are working. In this study, we describe a particular case in which animal keepers were involved in the administration of cyclophosphamide (CP) to mice.
CP is an agent widely used in hospitals, on one hand, as a cytostatic drug in cancer chemotherapy, and, on the other hand, as an immunosuppressant. CP is considered to be carcinogenic and mutagenic in humans and in animals.[1] During 1986-1988, the annual use in the Netherlands was about 56 kg.[2] Because of possible hazards due to occupational exposure to antineoplastic agents of hospital workers, extensive guidelines and safety precautions have been introduced.[3,4] Published studies about the occupational exposure to antineoplastic agents refer to workers in pharmacies, hospitals and industry.[5]
We have studied CP contamination in an animal room where mice were housed and treated with CP. Air and spot samples taken from different places, and subjects in different rooms in the animal laboratory were investigated for the presence of CP. Also contamination of masks, sleeve protectors, and gloves, used for protection, was studied. The uptake of CP by the animal keepers was measured by the determination of unmetabolized CP in urine. The present study is subdivided into two parts. In a first study, a former situation was investigated. Thereafter, some additional measures and safety precautions were taken that were evaluated in a second study.

Materials and methods

Study design

In the first and the second study, samples were taken during 7 (days 1-7) and 9 days (days 8-16), respectively. Three animal keepers (2 male, 1 female) were involved in the subcutaneous injection of CP to mice. A fourth animal keeper (male) cleaned the cages and the animal rooms. CP administration took place in one of the animal rooms where the mice were housed. Details about the amounts of CP administered to the mice are presented in Table 1. The animal keepers wore protective clothes and latex gloves. Almost always, masks and sleeve protectors were used.

Going from the central hall of the animal laboratory to the animal rooms, a sluice is entered where the animal keepers change their clothes and take other personal safety precautions. Thereafter, they enter a corridor that is connected with different animal rooms where animals were housed.

Table 1. Amounts of CP administered to the mice.

day	keeper	dose	number of mice	total dose (mg)
first study				
3	1	3.8	7	26.6
4	2[a]	3.8	50	190
5	3	2.5	50	125
6	4[b]	-[c]	-	-
7	1	2.5	100	250
second study				
9	3	3.7	30	111
10	3	2.5	30	75
11	3	-	30	-
12	4	-	-	-
13	3	3.7	60	222
14	2	2.5	60	150
15	3	3.7	100	370
16	1	2.5	100	250

[a] animal keeper 2 (female)
[b] animal keeper 4 was only involved in cleaning of the cages and the animal room
[c] -, CP was not administered

Air sampling

First study
Once on day 1, two stationary air samples were taken at the same time at different places in the animal room. Total airborne particulate matter was collected on polytetrafluoroethylene filters with 37-mm diameter and 1.2-μm pore size (Sartorius, Göttingen, Germany). In 5 hours, 0.63 m^3 of air was sampled (sampling rate 2.1 L/minute).

Second study
The air in the animal room was refreshed by an air-circulation system. When leaving the animal room, the air was sucked over two filters that were placed in the walls. The filters were changed weekly. Seven pair of filters were collected and analyzed for CP.

Wipe sampling
For the detection of contamination with CP, spot samples were taken before and after administration of CP and after cleaning of the cages and the animal room. In general, the spots were the same in the first and in the second study. Some spots were replaced by new spots. A description is given in Table 2. The method of sampling was performed like described previously.[6]

First study
On day 1, wipe samples were taken two times. The first time it was done for the estimation of "background" levels of contamination. The second sample was collected after cleaning of the animal cages in the animal room. Wipe samples were also taken after cleaning of the animal room on day 2 and before and after the administration of CP on day 3.

Second study
For the estimation of background levels of contamination, wipe samples were collected on the first day (day 8) also in this study. Before and after the administration of CP, wipe samples were taken during 4 days (days 9, 10, 13 and 14). This was also done on day 11, although no CP was administered. On day 16, wipe samples were only collected after the administration of CP.

Contamination of gloves, sleeve protectors, and masks

First study
During administration of CP and cleaning of the animal room, five pairs of latex gloves (Perry X-AM Latex Examination Gloves, Beaded Cuff, Smith & Nephew Perry, Massillon, Ohio) and five pairs of sleeve protectors (Tyvek, Hanotex, Sneek, The Netherlands) were worn. Surgery masks (Carpex Soft Face Masks, Beiersdorf AG, Hamburg, Germany) were used twice by animal keeper 1. After the administration of CP and after cleaning of the animal room, both gloves and both sleeve protectors were collected. For sample preparation left and right ones were put together. For the masks, the same procedure was followed.

Second study
Due to the results of the first study, during the injection of CP six double pairs of gloves and six pair of sleeve protectors were worn. The outer as well as the inner ones were collected separately. Animal keeper 4, who cleaned the cages, only used one pair of gloves and no sleeve protectors. All animal keepers wore masks except for animal keeper 3 (day 11).

Table 2. Description of the spots.

spot	description	area (cm^2)	limit of detection (ng/cm^2)
1	balance	520	0.8
2	working table	7600	0.02-0.1
3	tap	950	0.09-0.4
4	board above the washstand	850	0.5
5	latches of the door between the animal room and the corridor	400	1.0
6	floor in the animal room beneath the cages	4300	0.04-0.2
7	floor in the animal room beneath the cages	4300	0.04-0.2
8	floor in the animal room in front of the door	3500	0.05-0.2
9	floor in the corridor in front of the door of the animal room	3600	0.2
10	alcohol bottle	375	1.1
11	floor in the corridor (central position)	3300	0.05
12	latches of the door between the corridor and the sluice	300	0.3
13	floor in the sluice	3700	0.05
14	floor in the central hall	3200	0.05
15	service hatch	2400	0.07
16	phone	280	0.3
17	plastic foil on the working table	2400	0.07

Urine sampling
Total urine was collected in several portions during a period of 24 hours after the administration of CP or after cleaning of the cages and the animal room. They were stored at -20°C until sample preparation.

Sample preparation and CP determination
Sample preparation and CP determination with gas chromatography/mass spectrometry (GC/MS) was carried out according to Sessink et al.[6] Differences in detection limits between the first and the second study are caused by using a new GC column and changes in the parameter settings of the GC/MS during the second study.

Results

Air sampling

First study
No CP was found on the two filters of the stationary air samples. The limit of detection was approximately 1 $\mu g/m^3$.

Second study
On three pairs of filters of the air-circulation system, CP was found (Table 3). Because it was not possible to measure the amounts of air sampled, the amounts of CP per m^3 could not be calculated. Therefore, the amounts of CP were calculated per day, since all sampling periods were multiples of days and the activity of the circulation system could have changed during 24 hours (day-night) but not within days. The amounts of CP detected were between <0.1-1.0 μg/day.

Table 3. Amounts of CP on the filters of the air-circulation system.

day	samping period (days)	μg	μg/day
8	1	< 0 4	< 0 4
9	3	0 4	0 1
10	1	< 0 4	< 0 4
11	3	< 0 4	< 0 1
13	3	< 0 4	< 0 1
14	4	4 0	1 0
15	3	1 7	0 6

Wipe sampling

First study

On day 1, on almost all spots, background levels of CP were detected (Table 4). No CP was found on the latches and a part of the floor in the animal room beneath the cages. On day 2 and 3, CP was only detected on the balance, the working table, and the alcohol bottle.

Table 4. CP contamination of the working environment during the first study (ng/cm^2).

spot	day 1		day 2	day 3	
	series 1[a]	series 2[b]	series 3[c]	series 4[d]	series 5[e]
1	21	-[f]	1	-	-
2	8	4	1	2	2
3	2	nd[g]	nd	nd	nd
4	0.8	nd	nd	-	-
5	nd	nd	nd	-	-
6	nd	nd	nd	-	-
7	0.4	nd	nd	-	-
8	0.4	nd	nd	-	-
9	0.7	nd	nd	-	-
10	44	-	-	13	-

[a] samples taken for "background" levels
[b] samples taken after cleaning of the cages
[c] samples taken after cleaning of the animal room
[d] samples taken before administration
[e] samples taken after administration
[f] -, no sample taken
[g] nd, not detectable (for limits of detection, see Table 2)

Table 5. CP contamination of the working environment during the second study (ng/cm²).

spot	day 8	day 9		day 10		day 11		day 13		day 14		day 16
	series 6[a]	7[b]	8[c]	9[b]	10[c]	11[b]	12[c]	13[b]	14[c]	15[b]	16[c]	17[c]
2	nd[d]	nd	0.04	0.09	nd	nd	0.09	0.2	0.3	-[e]	nd	nd
3	nd	nd	nd	27	nd	nd	nd	nd	0.3	nd	nd	nd
6	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.04	nd	nd
7	nd	nd	0.05	nd	nd	nd	nd	nd	nd	0.04	nd	nd
8	nd	nd	nd	nd	nd	0.3	nd	nd	0.05	-	nd	nd
11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12	nd	nd	nd	-	6.4	6.9	nd	nd	0.6	nd	nd	nd
13	nd	nd	nd	nd	5.0	nd	nd	nd	nd	nd	2.4	nd
14	nd	nd	nd	nd	nd	0.7	nd	nd	nd	nd	nd	nd
15	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
16	-	-	-	-	-	-	nd	-	-	-	nd	-
17	-	-	10	-	0.8	-	nd	-	19	-	0.9	0.1

[a] samples taken for "background" levels
[b] samples taken before administration
[c] samples taken after administration
[d] nd, not detectable (for limits of detection see Table 2)
[e] -, no sample taken

Second study
On day 8, no detectable background levels were found (Table 5). On the other days, contamination was measured incidentally before and after the administration. The plastic foil on the working table was almost always contaminated. The highest amounts of CP per cm^2 were found on the tap, on the plastic foil on the working table, on the latches of the door between the corridor and the sluice, and on the floor in the sluice. No CP was detected on the floor in the corridor, on the service-hatch, and on the phone.

Contamination of gloves, sleeve protectors and masks

First study
During the administration of CP to the mice, CP was present on all gloves (Table 6). Glove contamination was not found for animal keeper 4 who was only involved in cleaning of the animal room. The sleeve protectors were contaminated once during the administration of CP. On the masks, no CP was detected.

Second study
Analysis of the samples of all outer pair of gloves showed the presence of CP (Table 6). CP was not found on the inner ones except for day 9 (animal keeper 3). On half of the sleeve protectors, CP was detected. No CP was found on the masks.

Urine samples
From the four animal keepers 87 urine samples were analyzed for CP. CP was detected in one urine sample of animal keeper 1 (Table 6). A total of 0.7 μg CP (in 205 mL of urine) was excreted over the period of 9-12 hours after CP injection. The limit of detection was approximately 1 ng/mL urine.

Discussion

In the present study, recently developed methods for the detection of environmental contamination with CP and for the measurement of the uptake of this compound by the determination of CP in urine were applied in a situation where animal keepers were possibly exposed to this chemical agent.[6,7] As far as we know, no studies, until now, have been published about the occupational exposure of animal keepers to CP or other antineoplastic agents. In general, little is known about the occupational exposure of this group of workers to chemicals, whereas it is known that, in animal laboratories, a lot of different chemicals are used.

Table 6. CP on the gloves, sleeve protectors, masks, and in the urine of the animal keepers (µg).

day	keeper	gloves[a]		sleeve protectors	masks	urine
		outer	inner			
first study						
3	1	23		< 7	< 3	nd[b]
4	2	75		< 7	-[c]	nd
5	3	19		< 7	-	nd
6	4	< 7		< 3[d]	-	nd
7	1	11		10	< 3	0.7
second study						
9	3	199	77	< 0.3	< 0.2	nd
10	3	47	< 0.3	< 0.3	< 0.2	nd
11	3	9	< 0.3	< 0.3	-	nd
12	4[e]	5	-	-	< 0.2	nd
13	3	12	< 0.3	0.6	< 0.2	nd
14	2	2	< 0.3	0.3	< 0.2	nd
16	1	36	< 0.3	0.4	< 0.2	nd

[a] only in the second study double pair of gloves were used
[b] nd: not detectable (different limits of detection depending on the urine volume)
[c] -, no sample taken (masks/sleeve protectors were not used)
[d] only one sleeve protector was collected
[e] only one pair of gloves was used

The results of the wipe samples taken in the first part of this study show that the animal room was contaminated with CP. Frequent contamination was found on the balance, on the working table and on the alcohol bottle. Before injection of CP, the mice were weighed on the working table. It is well known that mice urinate when they are seized. CP may be present in urine of CP-treated mice. The cages and the mice could be contaminated from previous CP administration, which possibly resulted in contamination of the different subjects and surfaces including the gloves. CP contamination was found on the alcohol bottle which was used frequently. This contamination could have resulted from contact with contaminated gloves. The results of the wipe samples also show that CP contamination was not only found in the animal room but also in adjacent rooms. In the stationary air samples, no CP was

detected, possibly because the volume sucked was too low. Nevertheless, it could be possible that small particulates were elevated from the cages that could be suspended in the air. Only in one urine sample (animal keeper 1) CP was detected. In spite of the use of protective clothes, gloves, sleeve protectors and masks, it is not clear whether the uptake occurred via inhalation, skin contact, or both. Ingestion is less probable because it was not allowed to take food or drinks.

In the first study, some spread out of CP contamination was found. Therefore, it was interesting to know if CP could be measured on other spots eg, in the corridor, the sluice, and in the central hall. Therefore, in the second part of the study, wipe samples were taken from these places. The results show that, not only in the animal room and in the corridor, but also in the sluice and in the central hall, CP contamination could be detected. Although the plastic foil on the working table became contaminated, the amounts of CP on the working table were diminished in comparison with the first observations. It is remarkable that the levels of CP in background samples were consistently higher. This may be caused by cumulation from the past in combination with bad routine cleaning. It may also be expected that, after informing the animal keepers about the results of the first study, they became more aware of the risks they may face. Therefore, they may have worked more properly during the second study, which resulted in lower environmental contamination. To check if CP could be measured in the air of the animal room, the filters of the air-circulation system were analyzed for CP. From the amounts measured it could be concluded that CP was present. Although the amount of air sampled could not be calculated, these amounts are supposed to be higher in comparison with the amounts of air sucked in the first study. CP contamination was found on the outer pair of gloves. The amounts measured are comparable with the amounts found in the first study. Except for the first sample, it is suggested that CP did not penetrate the outer gloves since no contamination was found on the inner ones. Taking the first sample, some complications with the collection of the gloves could have resulted in contact of the inner gloves with the outer ones. During the administration of CP, half of the sleeve protectors became contaminated. In comparison with the first study, this might be due to a lower detection limit. Despite the increase in sensitivity, CP could not be detected on the masks.

In the first study, CP was measured in urine of animal keeper 1. It is suggested that antineoplastic agents like CP are absorbed by inhalation and dermal penetration during preparation and administration of these drugs by pharmacy technicians and nurses.[8-10] Although the occupational activities in this study differ from the activities of hospital personnel, CP is able to penetrate the human skin.[9] This could be due to an inefficient protection by

gloves.[11-14]. In general, the degree of permeability appeared to be small, but lower permeability was found for gloves with higher thickness. The working period of animal keeper 1 was about 1.5 hours. Although the concentration of CP in the air was not measured during this working period (day 7), inhalatory uptake can be estimated by using the air concentration on day 1 (inhalatory uptake = air concentration x respiratory volume x working period x retention = < 1.0 $\mu g/m^3$ x 10 m^3/8 hours x 1.5 hours x 100% = < 1.9 μg). The amount of CP excreted as percentage of the inhalatory uptake is >37 (0.7 ug/<1.9 μg). Since a protective mask was used, it is expected that the real uptake will be lower. This percentage is much higher in comparison with animal studies and in studies with volunteers who received a low dose of CP. In animal studies were CP was dosed at 1 mg/kg, an excretion percentage of 5 was found.[15] After application of 1 mg on the skin of volunteers, about 1% was excreted unchanged in urine. These results do not agree with our estimation. When an equal percentage was excreted in our study, the uptake should be about 10 times higher. Therefore, we suggest that not inhalation, but dermal penetration, is the main exposure route.

Conclusion

In this study, it is shown that during the administration of CP to mice, in spite of certain safety precautions, CP contamination occurred. CP was found in urine of one of the animal keepers. The study demonstrates that animal keepers in this particular case are exposed to the chemical with wich they are working. Animal keepers may have contact with many different toxic chemicals. Knowledge about their possible exposure is lacking because of the absence of possibilities to measure exposure. The methods applied in this study seem suitable for the control of exposure to CP.

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2.5

Environmental contamination and assessment of exposure to antineoplastic agents by determination of cyclophosphamide in urine of exposed pharmacy technicians: Is skin absorption an important exposure route?

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Abstract

In the Netherlands, special guidelines and safety precautions were introduced about 10 y ago for preparation and administration of antineoplastic agents. However, little is known about the effectiveness of these measures. In this study, occupational exposure to antineoplastic agents of nine pharmacy technicians who were involved in drug preparation was investigated. Cyclophosphamide, 5-fluorouracil and methotrexate accounted for 95% of the antineoplastic agents prepared; therefore, the presence of these compounds was monitored. During preparation, cyclophosphamide was detected in the air of the work environment (<0.04-10.1 $\mu g/m^3$). Contamination of and permeation through the latex gloves used were found for each of the three compounds. The uptake of cyclophosphamide was assessed by the determination of cyclophosphamide in urine. The drug was found in urine samples of six pharmacy technicians, including three persons who were not directly involved in the preparation of cyclophosphamide. The amounts excreted ranged from 0.2 to 19.4 μg/24h. The results strongly suggest that inhalation is of minor importance for internal exposure, compared with other, presumably dermal, routes.

Introduction

During the handling of antineoplastic agents, pharmacy technicians and nursing personnel may face certain health risks[1]; therefore, special guidelines and extensive safety precautions have been introduced in the Netherlands[2,3] If the efficiency of these measures is to be studied, it is neccessary to monitor exposure levels and the possible uptake of these compounds by workers. Recently, we reported studies in which cyclophosphamide (CP), 5-fluorouracil (5FU), and methotrexate (MTX) contamination was detected in the work environment, and CP and iphosphamide (IP) were found in urine of nurses, pharmacy technicians,[4,5] and animal keepers.[6]
In this follow-up study, we were interested in whether CP, 5FU, and MTX were absorbed by inhalation or by dermal penetration. Personal and stationary air samples were taken during drug preparation in a hospital pharmacy department. Previous studies have shown that gloves used for protection during drug preparation become contaminated.[4,5] Other studies have demonstrated that some antineoplastic agents can permeate different types of gloves.[7-10] We investigated whether CP, 5FU, and MTX were able to permeate latex gloves usually worn during normal occupational activities, particularly during the preparation of the drugs. Uptake was determined by analysis of unmetabolized CP in urine.

Materials and methods

Study design

Samples were taken on 17 alternate d (Monday, Wednesday, and Friday) during 6 wk of drug preparation. Nine pharmacy technicians (eight women and one man; 22-35 y of age) were involved in the preparation; eight of these technicians had prepared drugs twice during this study. The drugs were prepared in a laminar downflow hood. Gloves, hair nets, and special clothes were worn for personal protection. Surgical face masks were worn by only one technician (no. 1).
The amounts of CP, 5FU and MTX prepared during this study were 14.55, 62.8, and 6.16 g, respectively. These compounds accounted for 95% of all antineoplastic agents prepared. For CP and MTX, vials with different amounts of drug were used. 5FU was packed in standard ampules of 250 mg.

Air sampling

During preparation of the drugs, stationary air samples were taken

approximately 30 cm from the technician and from the laminar downflow hood. Total airborne particulate matter was collected on polytetrafluoroethylene filters with 50-mm diameter and 1.0-μm pore size (Sartorius GmbH, Göttingen, Germany). The diameter of the cone was 20 mm, and the suction flow of the high volume sampler was about 23.5 l/min.
Personal air samples were taken with PAS-6. Total airborne particulate matter was collected on polytetrafluoroethylene filters that were 37-mm diameter and had a pore size of 1.2-μm. The suction flow was 2.0 l/min.

Glove contamination
One pair of white surgical gloves (style 42, Smith and Nephew Medical, Ohio, USA) was worn by each technician on each day of drug preparation. On the third day, however, one glove worn by one technician was torn and an additional one was used.
The permeation of the latex gloves used during preparation was determined by wearing cotton gloves (Laméris, Utrecht, the Netherlands) under the latex gloves. Except for technician 2, all technicians wore these cotton gloves on one occassion. After preparation of the drugs, the latex and the cotton gloves were collected separately. The cotton gloves were extracted and analyzed in the same manner as were the latex gloves.[5]

Urine sampling
Total 24-h urine was collected in portions, starting from the beginning of the drug preparation. Urine volumes and excretion periods were registered. Urine samples were stored at -20°C until sample preparation. Urine from three individuals employed in an administrative department served as nonexposed controls. The limit of detection was about 0.2 ng/ml urine.

Sampling procedures, sample preparation, and analysis
Sampling procedures, sample preparation, and analysis have been described elsewhere.[4,5]

Statistical analysis
Statistical analysis was carried out with SAS software version 6.06. Correlation was quantified with Spearman's rank correlation coefficient; p values refer to two-tailed tests. Half of the detection limit was used for nondetectable amounts.

Results

Air samples

No 5FU or MTS was detected on the stationary air sample filters. CP was only found on day 1 (Table 1). The limits of detection, depending on the amounts of the air sampled, ranged from 0.003 to 0.03 $\mu g/m^3$ for CP, from 0.2 to 2.2 $\mu g/m^3$ for 5FU, and from 0.5 to 5.4 $\mu g/m^3$ for MTX.

During personal sampling, CP was found on 4 d (Table 1). On day 8, CP was not prepared; 5FU and MTX were absent. The detection limits ranged from 0.04 to 0.5 $\mu g/m^3$ for CP, from 2.6 to 35 $\mu g/m^3$ for 5FU, and from 6.3 to 84 $\mu g/m^3$ for MTX.

Table 1. Stationary and personal air sampling of CP[a].

monitor type/day	technician	sampling time (min)	$\mu g/m^3$
stationary			
1	7	36	0.2
2-17	1-9	8-80	< 0.03
personal			
7	1	64	1.6
8	9	9	10.1
12	4	49	1.0
16	8	37	2.1
others	1-9	6-80	< 0.5

[a] 5FU and MTX were not detected

Glove contamination

Contamination of the latex and the cotton gloves was found frequently for 5FU and CP (Table 2). Both compounds were also present on the gloves when 5FU and CP were not prepared. MTX contamination of the latex gloves was found twice. In one case, it was also present on the cotton gloves. MTX was prepared during both days. The results show that CP, 5FU, and MTX can permeate the latex gloves. There is a correlation between the amounts of 5FU (on weight basis and number of ampules) prepared and the total (latex and cotton) glove contamination ($r = 0.68$, $p < 0.005$). No correlation was found for CP and MTX.

Table 2. Contamination with CP, 5FU, and MTX of gloves used during drug preparation (µg/pair).

day	technician	CP		5FU		MTX	
		latex	cotton	latex	cotton	latex	cotton
1	7	9.6	ns[a]	69	ns	< 20	ns
2	9	5.6	ns	< 4.0	ns	< 20	ns
3[b]	8	1.5	ns	190	ns	< 20	ns
4	6	9.4	ns	140	ns	< 20	ns
5	2	< 0.08	ns	21	ns	< 20	ns
6	6	< 0.08	15	< 4.0	< 4.2	< 20	< 11
7	1	< 0.08	ns	200	ns	< 20	ns
8	9	< 0.08	< 0.08	58	< 4.2	< 20	< 11
9	3	6.3	ns	26	ns	< 20	ns
10	5	< 0.08	ns	78	ns	< 20	ns
11	3	< 0.08	< 0.08	< 4.0	< 4.2	< 20	< 11
12	4	2.9	ns	240	ns	< 20	ns
13	1	< 0.08	14	< 4.0	290	< 20	< 11
14	4	< 0.08	73	620	450	1900	63
15	5	2.3	40	92	760	220	< 11
16	8	< 0.08	0.5	< 4.0	130	< 20	< 11
17	7	2.1	< 0.08	< 4.0	153	< 20	< 11

[a] ns: no sampling (cotton gloves were not used)
[b] tearing caused individual to use 3 gloves instead of one pair

CP in urine

CP was detected in the urine of five technicians (Table 3). The amounts excreted ranged from 0.2 to 19.4 µg (median = 0.6 µg). CP was found in the urine of technicians who did (n = 4) and who did not (n = 3) prepare CP. CP was present in the urine of technicians after the drugs were prepared with (n = 3) and without (n = 5) wearing the cotton gloves. No CP was detected in the urine of four technicians or the control urine samples.

Inasmuch as successive urine fractions were collected and analyzed, excretion of CP could be measured during the period of 24 h after the beginning of the preparation of the drugs. The cumulative CP excretion of the technicians who did and who did not prepare CP is presented in Figures 1 and 2, respectively. The figures show that for all workers, the excretion of CP started at different moments. CP was present mostly in successive fractions.

Table 3. Cumulative CP excretion (μg/24 h) in relation to the amounts of CP in the air and the amounts of CP prepared.

day	technician	amount of CP prepared (mg)	CP (μg) air[a]	CP (μg) urine
2	9	0	< 0.07	1.5
7	1	1200	2.2	19.4
8	9	0	1.9	1.5
9	3	1350	< 0.07	0.5
10	5	0	< 0.07	0.4
12	4	650	1.0	0.3
13	1	2100	< 0.07	0.7
14	4	0	< 0.07	0.2
16	8	3000	1.6	nd[b]

[a] calculated amount of CP "inhaled" during preparation
[b] nd: not detected

Discussion

In contrast to our previously mentioned studies, CP was detected also in air samples. This is surprising because the laminar downflow hood should not only protect the drugs prepared, but should also protect the technicians against inhalation of aerosols of released drugs. Release of aerosols of CP was also demonstrated by the presence of CP on two surgical face masks (4.3 and 350 μg; data not shown).

Sometimes the latex gloves were contaminated with a particular drug even though the drug was not prepared. This finding suggests that the contamination was already present. CP, 5FU, and MTX can permeate the latex gloves because these drugs were detected on the cotton gloves. In the normal work situation (without cotton gloves), this could result in dermal penetration, which was found to be the case for CP after application on the skin of volunteers by Hirst et al.[11]

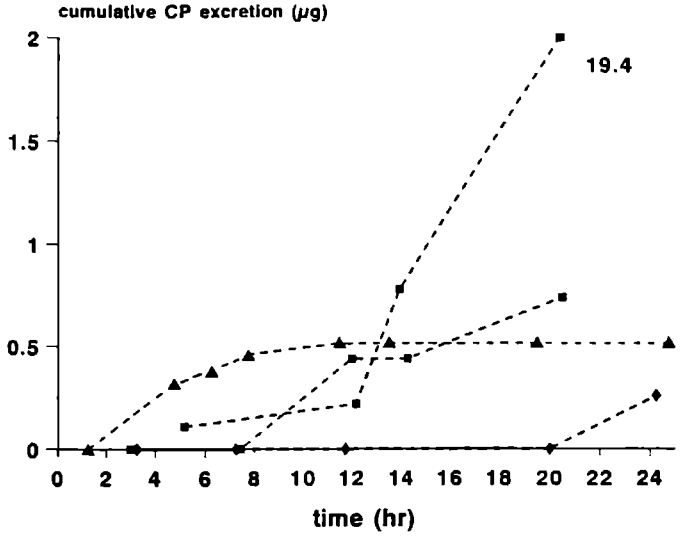

Fig. 1 Cumulative CP excretion of technicians preparing antineoplastic agents including CP Time was set at 0 when the preparation of the drugs started. (*Note* technician 1 = ■, technician 3 = ▲, and technician 4 = ◆)

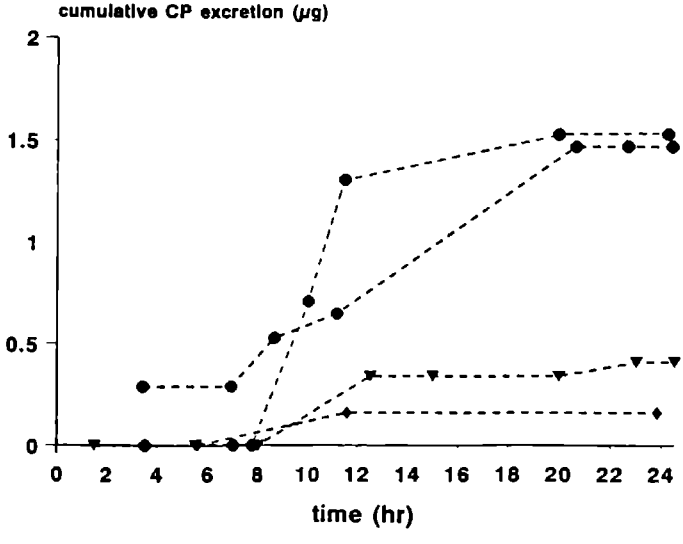

Fig. 2. Cumulative CP excretion of technicians preparing antineoplastic agents except for CP Time was set at 0 when the preparation of the drugs started (*Note* technician 4 = ◆, technician 5 = ▼, and technician 9 = ●)

The contamination of the cotton gloves, as percentage of the total contamination of both gloves, varied from 0% to 3% for MTX and from 0% to 100% for 5FU and CP. This suggests a low permeation for MTX and a high, but variable permeation for CP and 5FU. A reason for the difference in MTX permeation, compared with 5FU and CP might be the higher molecular weight and the polarity of MTX, both of which might prevent a quick permeation of the glove.

Another important factor is the time during which the gloves were worn because permeation increases with time.[8-10] Protection by latex gloves is afforded as long as no breakthrough is observed. The breakthrough time is defined as the time on which detectable amounts permeated the latex gloves and were found on the cotton gloves. With respect to CP, 5FU, and MTX, the earliest breakthrough times were 10, 37, and 62 min, respectively. These breakthrough times are much shorter than those reported by Colligan et al.[10] This might be caused by the lower detection limit of our analytical methods. Another reason might be that our model did not resemble the laboratory situation. In our study, it remains unknown what the influence of the cotton gloves was upon the permeation (rate) because "suction" could influence permeation, and flexing and stretching occurred when the latex gloves were used in combination with the cotton gloves. Nevertheless, according to Colligan et al.,[11] these effects should be negligible under laboratory conditions. It is remarkable that a correlation between the amounts of drugs prepared and the contamination of the gloves (latex plus cotton) was found for 5FU only. No such correlations were observed for CP and MTX. 5FU was packed in ampules, which had to be broken, and every break might have caused the release of some drug. The more 5FU prepared, the more (standard) ampules were broken, thereby releasing more 5FU. Vials with septa were used for MTX and CP, and for every amount used only one action was necessary.

The amounts of CP excreted in urine were higher, compared with results of previous studies.[4,5] In one study, no CP was detected.[4] In another study, the amounts ranged from < 0.01 to 0.5 μg (median = 0.1 μg).[5] In the present study, CP was present in the urine of technicians who did not prepare CP, which suggests environmental sources responsible for the uptake of this compound. In the previous studies, only in one or two urine fractions of one person was CP detected. In the present study, CP was present in more successive fractions or it was measured during different intervals, which resulted in higher total amounts excreted.

It is suggested that during occupational activities, CP is absorbed by inhalation or dermal penetration.[11-13] A quick excretion is ascribed to inhalation. On the other hand, dermal absorption will result in a postponed and slower excretion because of the skin barrier. The large differences in excretion patterns of

several technicians suggest that both exposure routes contribute to the occupational exposure of these workers. Nevertheless, it cannot be excluded that interindividual differences in toxicokinetic parameters also play a role.
The amounts of CP "inhaled" during drug preparation were calculated (Table 3). In these calculations, the concentration of CP in the air (Table 1) was multiplied by the sampling time (Table 1), a respiration volume of $10m^3$/8 h and a retention of 100%. The results show that the amounts of CP inhaled were generally lower when compared with the amounts of CP excreted. Also, as a result of biotransformation, it is expected that the amounts excreted are approximately 1-5% of the applied dose, which was found in CP-treated volunteers[11] and in CP-treated rats.[14]
The results show that the amounts of CP in urine cannot be explained by assuming inhalation to be the only exposure route. Instead, the results suggest that other exposure routes, in addition to inhalation, are involved. Whether this is dermal penetration remains unknown. However, the combination of easy permeation of CP through the latex gloves and the possibility of skin penetration make it reasonable to assume that dermal uptake is an important exposure route.

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2.6

Exposure of pharmacy technicians to antineoplastic agents; reevaluation after additional protective measures

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Submitted

Abstract

Special guidelines and protective measures have been introduced to protect hospital workers during the handling of antineoplastic agents. Nevertheless, it was found that they do not prevent the uptake of these toxic compounds. Therefore in this Dutch hospital additional protective measures have been introduced. The measures included adaptations of the laminar downflow hood, the use of special masks and double pairs of gloves by the workers and the replacement of ampules in vials. The effects of these additional measures were compared with the results of a previous study. As cyclophosphamide (CP), 5-fluorouracil (5FU), and methotrexate (MTX) comprised 81% of the antineoplastic agents prepared, these compounds were monitored again by personal air-sampling and the determination of contamination of masks and gloves. Biological monitoring was performed by the determination of CP in urine of the workers. During preparation, lower concentrations of CP in the air were observed in comparison with our previous study. Replacement of ampules by vials (5FU) resulted in a significantly diminished contamination of the latex gloves with 5FU. CP was detected in urine samples of six of nine technicians. The maximum amount excreted over five days was 2.6 μg. The mean CP excretion per day was not significantly lower, than in the previous study (0.16 μg and 1.44 μg respectively). In spite of an intensified hygienic regimen, exposure to antineoplastic agents can not be reduced as long as the reasons for the exposure are unknown.

Introduction

In The Netherlands new special guidelines and protective measures were introduced about 10 years ago to protect hospital workers for the health risks of handling of antineoplastic agents.[1] Recently, these measures were amended.[2] Other countries also have protective guidelines.[3-6] Nevertheless, several studies have shown uptake of antineoplastic agents in hospital workers following guidelines.[7-15]

Our recent study of the effectiveness of the protective measures showed that during preparation, cyclophosphamide (CP) was detected in the air of the working environment at concentrations of <0.04-10.1 $\mu g/m^3$.[7] Contamination of, and permeation through latex gloves were found for the three drugs studied: CP, 5-fluorouracil (5FU) and methotrexate (MTX). In addition, CP was detected in urine samples of some workers. The results showed that although protective measures were taken, exposure to these compounds still occurred. More strongly, the protective measures were not able to prevent the uptake of CP. It was suggested that inhalation was of minor importance for internal exposure, compared with other, presumably dermal, routes.

These results led in this particular hospital to the introduction of additional protective measures. The working aperture of the laminar downflow hood was reduced by enlargement of the viewing screen. It was also decided that the pharmacy technicians should use double pairs of gloves and a special mask. Besides, vials with 5FU were used for preparation instead of ampules. In a follow-up study, the effects of these additional measures were investigated. Personal air-samples were taken and analyzed for the presence of CP, 5FU and MTX. Permeation of latex gloves was studied by analyzing the contamination of both the inner and the outer gloves. The uptake of CP was determined by analysis of unmetabolized CP in urine.[16] The results of this study were compared with the results of the previous study.

Materials and methods

Study design

Nine pharmacy technicians were involved in drug preparation. Eight of them were also involved in the previous study.[7] Their codes were the same. The drugs were prepared in a laminar downflow hood during five successive days (Monday to Friday). For personal protection, latex gloves, hair-nets and special clothes were used. In contrast with the previous study, all technicians used a double pair of gloves and a special mask. The technicians were aware of the

new study design and monitoring. Any possible effect on behaviors is not expected because they continued working on the same way. The amounts of CP, 5FU and MTX prepared during this study were 24, 195, and 5 g, respectively (Table 1). These compounds contributed to 81% of the antineoplastic agents prepared. CP, 5FU and MTX were packed in vials.

Air sampling

Personal air sampling was performed identical as described before.[7] The samples were taken during five successive days from all nine technicians.

Masks

The masks (Filgif, super sigma 5005-V mask, Groeneveld-Dordrecht B.V., Dordrecht, The Netherlands) were collected after drug preparation in such a way that no contamination with other materials like for instance the gloves was possible. They were packed in aluminum foil until sample preparation.

Glove contamination

During all days the technicians wore double pairs of gloves (Ansell Gammex latex sterile surgical gloves, Ansell Medical, München, Germany). After preparation of the drugs the pairs of gloves were collected separately.

Urine sampling

Total urine was collected in portions during five days starting at the beginning of the preparation of the drugs. The urine volumes and the excretion periods were registered. The urine samples were stored at -20°C until sample preparation.

Sampling procedures, sample preparation and analysis

Sampling procedures, sample preparation and analysis were described elsewhere.[8,9,16] The masks were cut in pieces and extracted by sonication during 90 min and subsequent shaking during 10 min with 250 ml of a 0.03 M sodium hydroxide solution. The extracts were used for direct analysis (5FU and MTX) or further clean up (CP).

Statistical analysis

Statistical analysis was carried out with SAS software version 6.06. Correlation was quantified with Spearman's rank correlation coefficient; p values refer to two tailed tests. Only p values equal to or below 0.05 were considered to be significant. Half of the detection limit was used in case of non-detectable amounts.

Table 1. Amounts of CP, 5FU and MTX prepared (mg)[a].

technician	day	CP	5FU	MTX
1	Mon	900	2600	0
	Tue	650	1100	0
	Wed	0	38000	0
	Thu	0	0	75
	Fri	0	1000	30
2	Mon	1250	1100	0
	Tue	1000	0	0
	Wed	500	0	0
	Thu	0	25000	0
	Fri	0	0	0
3	Mon	600	4500	0
	Tue	600	0	0
	Wed	0	1600	0
	Thu	600	0	0
	Fri	0	25900	0
5	Mon	1200	19900	0
	Tue	0	4950	30
	Wed	1150	0	0
	Thu	600	0	0
	Fri	0	900	0
6	Mon	1000	5900	0
	Tue	650	0	0
	Wed	550	0	0
	Thu	900	900	0
	Fri	0	17900	0
7	Mon	0	4800	0
	Tue	1200	0	0
	Wed	0	0	0
	Thu	0	0	0
	Fri	0	1500	15
8	Mon	250	800	0
	Tue	900	1100	0
	Wed	400	0	0
	Thu	0	0	75
	Fri	0	500	60
9	Mon	650	800	0
	Tue	3000	900	0
	Wed	0	0	4910
	Thu	400	25000	0
	Fri	1200	900	0
10	Mon	1000	6700	0
	Tue	350	0	0
	Wed	1600	0	0
	Thu	900	900	75
	Fri	300	0	30
total		24300	195150	5300
percentage[a]		9	70	2

[a] as perentage of all antineoplastic drugs prepared

Results

Air samples

On the filters of the personal air samples no 5FU and MTX were detected. CP was found on two days. The air concentrations were calculated to be 0.06 (technician 2 on Wednesday) and 2.0 $\mu g/m^3$ (Technician 8 on Tuesday). On both days CP was prepared. The limits of detection, depending on the amounts of air sampled, ranged from 0.02-0.15 $\mu g/m^3$ for CP, 1.6-9.9 $\mu g/m^3$ for 5FU and 3.8-24 $\mu g/m^3$ for MTX.

Masks

Ten out of forty five masks were contaminated with CP (Table 2). The amounts ranged from <0.2-8.8 μg. CP was present on two masks whereas it was not prepared. The level of contamination was not correlated with the amount of drug prepared and the number of vials used. One mask was contaminated with 15 μg 5FU while no masks were contaminated with MTX. No drugs were detected on masks of five technicians. The limits of detection per mask were 0.2 μg for CP, 5 μg for 5FU and 13 μg for MTX.

Table 2. Contamination with CP and 5FU of masks used during drug preparation[a].

technician	day	contamination (μg)	
		CP	5FU
1	Mon	5.5	<5
	Tue	2.2	<5
	Wed	<0.2	15
5	Mon	0.20	<5
	Thu	0.28	<5
	Fri	8.8	<5
8	Mon	2.0	<5
	Tue	1.8	<5
	Thu	1.8	<5
9	Tue	6.2	<5
	Fri	5.7	<5

[a] only masks with detectable amounts of one of the drugs measured are indicated

Glove contamination

Contamination of the outer pairs of latex gloves was found most frequently for CP (twenty six pairs of gloves) and 5FU (nine pairs of gloves) (Table 3). The amounts ranged from <0.13-140 μg (median: 1.2 μg) for CP and from <2.0-450 μg (median: <2 μg) for 5FU. One outer pair of gloves was contaminated with 94 μg MTX (Technician 9 on Wednesday). 5FU was present on one outer pair of gloves when no preparation of 5FU occurred. For CP this was observed for three pairs of gloves. The level of contamination of the outer pairs of gloves was significantly correlated with the amount of drugs prepared (CP: $r=0.60$, $p=0.0001$; 5FU: $r=0.32$, $p=0.03$) and the number of vials used (CP: $r=0.63$, $p=0.0001$; 5FU: $r=0.47$, $p=0.001$). Contamination of the inner pairs of latex gloves was only found for CP (seven pairs of gloves) (Table 3). The amounts ranged from <0.13-9.1 μg (median: <0.13 μg). CP was found on one inner pair of gloves when it was not prepared. The level of contamination of the inner pairs of gloves was not significantly correlated with the amount of drugs prepared and the number of vials used. The contamination with 5FU of the inner latex gloves with 5FU could not be quantified because of interference of coeluting compounds during analysis of the samples. The limits of detection per pair of gloves were 0.13 μg for CP, 2 μg for 5FU and 10 μg for MTX.

CP in urine

CP was detected in urine samples of six technicians (Table 4). The maximum amount excreted over five days was 2.6 μg. All technicians prepared CP during at least one day (mean: three days). No correlation was found between the total amounts excreted over five days and the amounts of CP prepared. Even when the amounts of CP prepared were averaged by the number of days of preparation. The limit of detection was about 0.25 ng/ml urine.

Discussion

In a previous study CP was detected in personal air samples during four out of seventeen days.[7] The air concentrations ranged from 0.04-10.1 $\mu g/m^3$. In the present study CP was detected once again although less frequently (two out of forty five days) and the concentrations were significantly lower (Wilcoxon test: $p=0.005$). Like in the previous study, also in this study no 5FU and no MTX was detected in personal air samples. Adaptations of the laminar downflow hood may be responsible for the observed decrease in CP air concentrations. Obviously, the release of aerosols could not be totally prevented which was also demonstrated by the presence of CP on the masks.

Table 3. Contamination with CP and 5FU of gloves used during drug preparation[a].

technician	day	CP contamination (μg/pair)		5FU contamination (μg/pair)
		outer gloves	inner gloves	outer gloves
1	Mon	4.1	<0.13	14
	Tue	3.7	<0.13	<2
	Wed	<0.13	2.4	9.1
2	Mon	1.9	<0.13	<2
	Tue	<0.13	<0.13	26
	Wed	0.37	<0.13	<2
3	Mon	54	<0.13	<2
	Tue	1.7	<0.13	<2
	Thu	2.1	<0.13	<2
	Fri	<0.13	<0.13	450
5	Mon	83	1.4	12
	Wed	11	<0.13	<2
	Thu	5.9	<0.13	<2
6	Mon	<0.13	<0.13	38
	Tue	0.27	<0.13	<2
	Thu	30	<0.13	<2
	Fri	2.6	<0.13	200
7	Mon	<0.13	<0.13	37
	Tue	2.9	<0.13	<2
8	Mon	2.4	1.3	<2
	Tue	2.3	<0.13	<2
	Wed	1.3	1.5	<2
	Thu	1.2	<0.13	<2
9	Mon	4.5	<0.13	<2
	Tue	70	<0.13	<2
	Wed	3.4	<0.13	<2
	Thu	5.4	3.2	<2
	Fri	140	9.1	17
10	Mon	0.73	<0.13	<2
	Tue	<0.13	1.3	<2
	Wed	1.4	<0.13	<2
	Fri	1.8	<0.13	<2

[a] only gloves with detectable amounts of one of the cytostatic drugs measured are indicated

Table 4. Cumulative CP excretion in urine of pharmacy technicians and the amounts of CP prepared.

technician	CP prepared (mg)	CP in urine (μg/five days)
1	1550	0.6
2	2750	nd[a]
3	1800	nd
5	2950	2.1
6	3100	0.2
7	1200	nd
8	1550	0.6
9	5250	1.1
10	4150	2.6
all	24300	7.2

[a] nd: not detected

MTX was not detected on the masks whereas the presence of 5FU was observed once after the preparation of a large amount of 21000 mg of 5FU.
In both the present and the previous study contamination with MTX of the outer latex gloves was hardly observed. In the present study it was found once when a large amount of 4910 mg of MTX was prepared. In the previous study contamination with 5FU of the outer latex gloves was found during eleven out of seventeen days (range: <4.0-620 μg) whereas in the present study 5FU contamination was observed during nine out of forty five days (range: <2.0-450 μg). Both, the frequency of contaminated outer gloves (Fisher's exact test $p=0.04$) and the level of contamination (Wilcoxon test $p=0.001$) were significantly decreased. Whether the diminished contamination of the outer gloves with 5FU was due to the use of vials instead of ampules needs further investigation. Possibly the breaking of ampules is a cause for the release of aerosols resulting in contamination of the gloves.[7]
During the previous study contamination with CP of the outer latex gloves was found during eight out of seventeen days (range: <0.08-9.6 μg).[7] During the present study contamination with CP was observed during twenty six out of forty five days (range: <0.13-140 μg). No significant differences were found between the frequency of contaminated outer gloves and the level of contamination in the present and the previous study. Considering an important role for the breaking of ampules, this is not surprising because in both studies

vials were used for CP preparation. In the previous study cotton gloves were worn under the latex gloves to study the permeation through the latex gloves for CP, 5FU and MTX. In the present study the cotton gloves were replaced by latex gloves identical to those used as outer gloves. Also in the present study, permeation through the outer latex gloves was found. In the previous study five out of eight pairs of cotton gloves were contaminated with CP (range: <0.08-73 μg). From four of these contaminated pairs of cotton gloves the corresponding outer pairs of latex gloves were not contaminated with CP suggesting 100% permeation of CP. In the present study CP was detected on seven out of forty five inner pairs of latex gloves (range: <2-9.1 μg). No CP was found on the corresponding outer pairs of latex gloves from two of these contaminated pairs of gloves. No correlation was found between the level of contamination on the inner latex gloves and the amount of CP prepared or the number of vials used. However, the sum of the amounts of CP contaminated on the outer and the inner latex gloves was significantly correlated with the amount of CP prepared ($r=0.56$, $p=0.0001$) and the number of vials used ($r=0.60$, $p=0.0001$). The level of CP contamination on the inner latex gloves was significantly diminished in the present study when compared to the previous study (Wilcoxon test $p=0.02$). Because no difference was observed between both studies concerning the frequency of contaminated outer gloves and the level of contamination with CP, it is plausible to suggest that the diminished contamination was caused by the use of latex gloves as inner gloves.

In the previous study in 24 hr urine samples of five out of nine workers CP was detected.[7] In the present study in urine samples of six out of nine workers CP was found, although it should be noticed that these samples were collected over a longer period (five days versus one or two days). Mean amounts of CP per day were calculated and the results were compared with the mean amounts of CP per day calculated from the previous study. A decrease from 1.44 to 0.16 μg in mean CP excretion per day was found. Although the difference is big, it is not significant (paired Wilcoxon test).

The results show that the introduced additional protective measures have reduced the external exposure to CP and 5FU. MTX was hardly measured which is possibly due to a higher detection limit of the analytical method. To what extent each of the additional protective measures has reduced this decrease, remains unknown. Nevertheless, it is shown that the contamination of the latex gloves is reduced possibly due to the use of vials instead of ampules. Besides, the results of the filters of the personal air samples show that at this moment CP, 5FU and MTX were hardly found in the air of the working environment. It is a question whether the use of double pairs of latex gloves has diminished the permeation of CP. It is possible that the

contamination with CP was decreased due to permeation of CP through the inner pairs of latex gloves. This might have resulted in skin contact and hence absorption.
The final aim of this study was to investigate whether these additional protective measures have reduced the uptake of CP. It should be noticed that a 90% reduction in exposure levels is important even if not significant. There might be several reasons for the absence of significancy between both studies concerning the amounts of CP in urine. The absence of significance is probably caused by large S.D.'s due to large interindividual differences; even the individual values of the workers were paired between both studies. Absence of significance is probably also a consequence of the small group of workers ($n = 8$). In addition there is no reason to expect high correlations with external dose when wide coefficients of variation for low exposure levels are observed. Nevertheless, the amounts of CP excreted in urine in this study are more comparable with the results of urinary CP excretion in previous published studies dealing with other groups hospital pharmacy workers who are working under situations with comparable protective measures.[9,17]
The question whether exposure can be reduced by reducing use is a difficult one. Normally, it is reasonable to assume a possitive correlation between use and exposure. It is a question to what extent diminished use will result in diminished exposure because it is not clear at all how exposure takes place. The presence of environmental contamination may result in chronic exposure. We expect that diminished use will not reduce the environmental contamination at that moment present in the working area. The results of this study indicate that in spite of efforts in a hygienic regimen exposure to antineoplastic agents can not be reduced. Only when we exactly are aware of the relationship between sources of exposure, distribution and absorption, we may be able to reduce the level of exposure to antineoplastic agents any further.

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2.7

Assessment of occupational exposure of pharmaceutical plant workers to 5-fluorouracil. Determination of α-fluoro-ß-alanine in urine

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Abstract

The exposure of pharmaceutical plant workers, involved in drug compounding and drug production, to 5-fluorouracil (5FU) was studied. 5FU was found by the analysis of air and wipe samples. During weighing, 5FU was detected in the air (75 $\mu g/m^3$). 5FU was also present on the filter of the mask of the weigher (120 μg). Before drug compounding 5FU was found on the floor (range, <1 to 8 ng/cm^2; median, 2 ng/cm^2). After routine cleaning significant higher amounts of 5FU were measured (range, 70 to 630 ng/cm^2; median, 150 $\mu g/cm^2$; $P=0.02$). The amounts of 5FU present on several objects were lower when compared to the amounts on the floor. The gloves used were always contaminated (range, 22 to 720 μg/pair of gloves; median, 141 μg/pair of gloves). The uptake of 5FU was established by the determination of α-fluoro-ß-alanine (FBAL), the main metabolite of 5FU, in the urine of the workers. Fifty micrograms of FBAL were found in urine of the weigher.

Introduction

In the Netherlands, 5-fluorouracil (5FU) is the most frequently used antineoplastic agent for parenteral administration. Its annual use over 1986 to 1988 was about 35 kg.[1] 5FU belongs to the group of the antimetabolites. The

results of an animal study for assessing the possible carcinogenicity of 5FU were considered to be inadequate.[2-4] Neither chromosome aberrations nor sister chromatid exchanges were induced in peripheral blood lymphocytes of patients treated with 5FU. 5FU induced micronuclei but not specific locus mutations in mice treated in vivo. It induced aneuploidy, chromosomal aberrations and sister chromatid exchanges in cultured Chinese hamster cells. It did not induce sex-linked recessive lethal mutations in Drosophila, but caused genetic crossing-over in fungi. Studies in bacteria were inconclusive. 5FU is found to be embryotoxic in animals.

5FU is quickly metabolized in rodents and man.[2] After reduction in the liver, ring opening resulted in the formation of ammonia, carbon dioxide, urea, α-fluoro-ß-alanine (FBAL) and fluoride. In a patient treated with 5FU, about 80% of the dose is quickly excreted in urine as FBAL.[5]

For measuring occupational exposure to antineoplastic agents different methods have been developed in our laboratory which are now validated in practice.[6-9] In the present study, environmental monitoring was carried out by taking air samples followed by the analysis of the samples for the presence of 5FU. To establish the uptake of 5FU in workers occupationally exposed to this compound, only compound specific detection methods can be used since 5FU is not mutagenic and cytogenetic effects are negligible.[2-4] Because it is expected that the exposure to 5FU will be low (because of safety precautions) and 5FU is quickly metabolized, it is reasonable to assume that the amounts of 5FU in urine are too low to be detectable. Therefore, a chemical analytical procedure for the detection of FBAL in urine was developed. Until now only high amounts of FBAL (1 mg/L) could be detected in patients using ^{19}F-nuclear magnetic resonance.[5] For low amounts this method may not be suitable. Therefore a new sensitive method was developed. After derivatization with S-ethyltrifluorothioacetate and acidified n-butyl alcohol, FBAL was determined using gas chromatography/mass spectrometry (GC-MS).[10] This GC-MS method, in combination with the environmental analysis of 5FU (air and wipe samples of possibly contaminated surfaces and objects, contamination of the gloves and the filters of the masks), was validated in a pharmaceutical plant where workers compound and produce 5FU.

Materials and methods

Manufacturing process

In the pharmaceutical plant, the manufacturing process of 5FU was subdivided into two steps: 1) drug compounding and 2) drug production, which took place during two successive days in different overpressure rooms. During drug

compounding (day 1) about 3.5 kg of 5FU was taken out of a barrel, weighed, and mixed in a reactor with water, sodium chloride, and sodium hydroxide. After that, the solution was pumped via a filter from the drug compounding room to the aseptic production room where the vials were filled (day 2). Two workers were involved in drug compounding (day 1): a weigher and a controller. The weigher weighed the chemicals, including 5FU, and the controller managed the compounding process. During drug compounding, special protective clothes were worn. A hair net was used and the shoes were covered. Gloves and masks with special filters were worn from the moment 5FU was weighed. Three workers were involved in drug production (day 2): a controller (same person as on day 1), an operator, and a packer. The controller coupled the barrel with the filtered solution to the production machine which filled the vials. The operator runned the production process and cleaned the machine when vials were broken. The controller and the packer removed the broken vials from the machine. The packer placed the sealed vials in boxes. Also during drug production special protective clothes, gloves and boots were worn. Except for the eyes, the faces of the workers were totally covered.

Air sampling

During drug compounding (about 3 hours), a stationary air sample was taken with a high volume sampler which was placed near the balance.[11] Total airborne particulate matter was collected on a cellulose filter with 47 mm diameter and 1.0 μm pore size (type RC 60, Schleicher & Schuell, 's-Hertogenbosch, The Netherlands). Air (8.9 m^3) was sampled (sampling rate, 50 L/min). The limit of detection was about 0.2 $\mu g/m^3$.

During weighing of 5FU (about 1 hour), personal air samples were taken with PAS-6.[12] Total airborne particulate matter was collected on polytetraethylene filters with 37 mm diameter and 1.2 μm pore size (Sartorius GmbH, Göttingen, Germany). Air (120 L) was sampled (2.0 L/min, 1.25 m/sec). The limit of detection was about 15 $\mu g/m^3$.

Wipe sampling

Wipe samples were taken from the floor of the drug compounding room before the beginning of the drug compounding process and after routine cleaning. The wipe samples were taken from the floor beneath and in front of the balance and in the center and beneath the sink unit. In the corridor, which connected the sluice with the drug compounding room, an additional sample was taken.

Wipe samples were also taken from different objects which were placed in the drug compounding room like the balance, including the table and the screen, the lid of the waste bin, and the latch of the door between the drug

compounding room and the sluice. The detection limit for the wipe samples was 3.5 μg.

Gloves and mask filters

The two filters of the masks worn during drug compounding (about 1 hour) were analyzed for contamination with 5FU. The limit of detection was 7 μg/filter.

A single pair of gloves (Ansell non-powdered sterile latex hypoallergenic surgeon's gloves, Ansell Medical, Munich, Germany) were worn during drug compounding and during drug production. During drug compounding, the weigher and the controller wore two single pairs of gloves. During drug production, the controller used three gloves (because of tearing, an additional one was used). The operator and the packer wore two single pairs of gloves. Both gloves of each pair were collected together. The limit of detection was 14 μg/pair of gloves.

Urine sampling

Total urine was collected in portions during the period of about 24 hours starting from the beginning of the working day. The amounts excreted and the excretion periods were registered. The urine samples were stored at -20°C until sample preparation.

Chemicals

5FU (purity >99%) was obtained from Janssen Chimica (Beerse, Belgium) and FBAL (purity >99%) was purchased from Interchim (Montluçon, France). *S*-Ethyltrifluorothioacetate (purity >99%) was obtained from Sigma Chemical Company (St Louis, MO). All other chemicals were of the highest purity obtainable.

Sampling procedures, sample preparation and HPLC analysis of 5FU

Sampling procedures, sample preparation and HPLC analysis of 5FU in air- and wipe samples and on the gloves were performed according to Sessink et al.[6-9] For the filters of the masks, the same procedures were followed as for the gloves. The limit of detection for 5FU was about 65 μg/L extract.

GC-MS analysis of FBAL in urine

Sample preparation and derivatization

One milliter of urine and 100 μl of a 5 mol/L sodium hydroxide solution were combined in tubes with screw caps. After addition of 150 μl of S-ethyltrifluorothioacetate, the tubes were tightly closed and shaken for 5 hours

at room temperature.[10] One hundred microliters of concentrated hydrochloric acid were added and the solution was mixed. The samples were extracted twice with 3 mL of ethylacetate. The combined ethylacetate layers were dried under nitrogen at 50°C. After addition of 0.5 mL of freshly prepared acidified n-butyl alcohol (250 μL of acetylchloride in 5 mL of n-butyl alcohol) and subsequent mixing, the tubes were closed for butylation for 1 hour at 90°C. The samples were cooled down to room temperature and dried under nitrogen at 40°C. After that, 250 μL of toluene were added and vials were filled with sample and stored at -20°C until analysis.

GC-MS conditions

GC-MS analysis was performed on a Varian Saturn GC-MS system controlled by a Compaq 386-20e personal computer. The on-column injection mode was used (SPI: Septum equipped temperature Programmable Injector). A 30-m DB-5 column (J & W Scientific, Folsom, CA) was used with 0.25-mm internal diameter and 0.25-μm film thickness. The column was connected with a deactivated fused silica retention gap (Varian, Houten, The Netherlands) with a length of 5 m and an internal diameter of 0.53 mm. The initial injector temperature was 110°C. After 1 minute the temperature was increased by 180°C/min to 280°C. After 15 minutes at 280°C the temperature was decreased to the initial temperature by cooling with liquid carbon dioxide. The initial oven temperature was 110°C. After 1 minute, the temperature was increased by 75°C/min to 140°C, and thereafter by 20°C/min to 280°C, where it remained constant for 7 minutes. Helium was used as carrier gas (column inlet pressure, 14 psi). The interface temperature was 280°C and electron impact was used as ionization mode. Identification was carried out by the combination of full scan spectra (50-399 atomic mass units) and the retention time of derivatized FBAL, which was 5.0 minutes.

Quantification was performed on the selected ion fragment 186, which was abstracted from the full scan spectra. For quantification the peak area of derivatized FBAL was calculated. Quantification of the butylated trifluoroacetyl derivative of FBAL was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference urine samples containing FBAL. Linearity was found until at least 50 mg/L ($r > .99$). The limit of detection was about 60 ng/mL urine (signal:noise ratio > 4).

Results

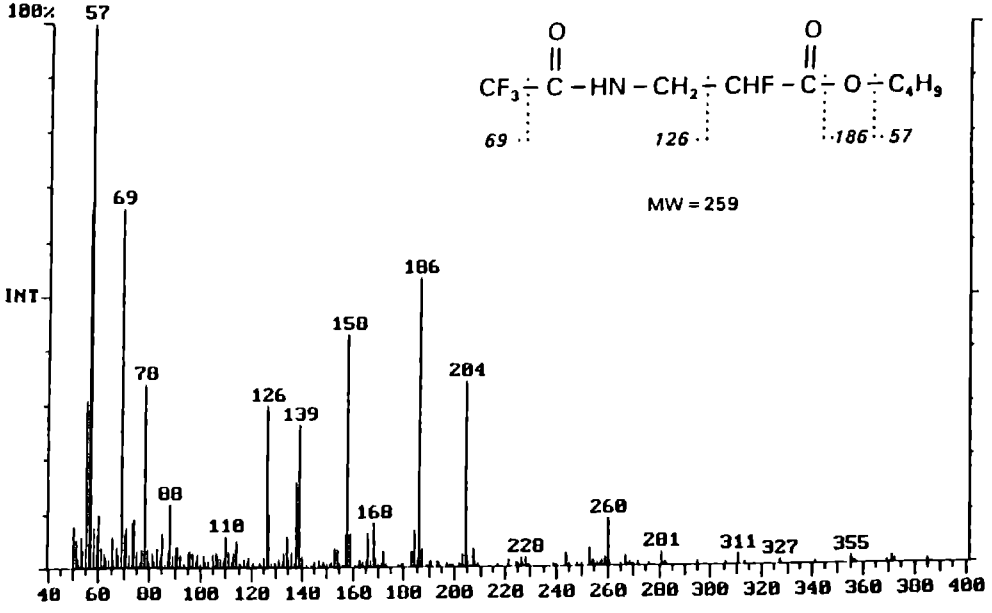
Sample preparation, derivatization and GC-MS analysis of FBAL

We have developed an efficient and quick method to carry out the clean-up

procedure routinely. FBAL is acetylated with S-ethyltrifluorothioacetate under basic conditions.[10] After acidification, the dried extracts were derivatized with freshly prepared acidified n-butyl alcohol. The described procedure is rather simple and suitable for routine analysis.

The mass spectrum of derivatized FBAL (Fig. 1) is in concordance with the mass spectrum of its analog ß-alanine as reported by Leimer et al.[13] For quantification it was not possible to use the molecule fragment 259 because this fragment is not stable under electron impact conditions, consequently, a low relative intensity was observed. The high relative intensity in combination with a general high specificity for the higher ion fragments resulted in the choice of ion fragment 186 for the quantification of FBAL.

Fig. 1. Mass spectral fragmentation of N-trifluoroacetyl-n-butyl ester of FBAL.



Manufacturing process

5FU was not detected in the stationary air sample (<0.2 $\mu g/m^3$). In the personal sample of the weigher, 5FU was present (75 $\mu g/m^3$). In the personal sample of the controller, no 5FU was detected (<15 $\mu g/m^3$).

Before the beginning of drug compounding, low amounts of 5FU were detected on the floor of the drug compounding room (Table 1). It is remarkable that the amounts of 5FU after routine cleaning were significantly higher than before drug compounding ($P=0.02$, Mann-Whitney U test). The highest amounts were found beneath the balance. Outside the drug compounding room, low amounts of 5FU were detected on the floor in the corridor. The different objects studied were also contaminated with 5FU, although the amounts were lower when compared to the amounts measured on the floor (Table 2). On the filter of the mask of the weigher, 120 μg 5FU were detected. No 5FU was found on the filter of the mask of the controller (<7 μg). During all activities the gloves of all workers became contaminated with 5FU (Table 3).

In one urine fraction of the weigher (780 mL), 50 μg of FBAL were detected that was excreted during the period of 10 to 13.75 hours after the start of weighing 5FU (Fig. 2). In the urine fractions of the controller, operator, and packer no FBAL was detected (<60 ng/mL).

Table 1. Contamination of the floor of the drug compounding room with 5FU (ng/cm^2).

floor description (cm x cm)	before drug compounding	after routine cleaning[a]
beneath the balance (70 x 50)	2	490
beneath the balance (80 x 50)	2	630
in front of the balance (80 x 60)	8	110
in front of the balance (80 x 80)	3	150
in the center (130 x 30)	< 1	70
beneath the sink unit (95 x 50)	50	ns[b]
beneath the sink unit (95 x 50)	< 1	ns
corridor (80 x 60)	2	< 2

[a] statistically different from before drug compounding ($P=0.02$, Mann-Whitney U test)

[b] ns, no sampling

Table 2. Contamination of the objects in the drug compounding room with 5FU (μg).

object	before drug compounding	after routine cleaning
balance table	nd[a]	22
inside balancescreen (left)	11	ns[b]
inside balancescreen (right)	nd	ns
balance	ns	50
lid of the waste-bin	ns	50
latch door between compounding room and sluice	ns	ns

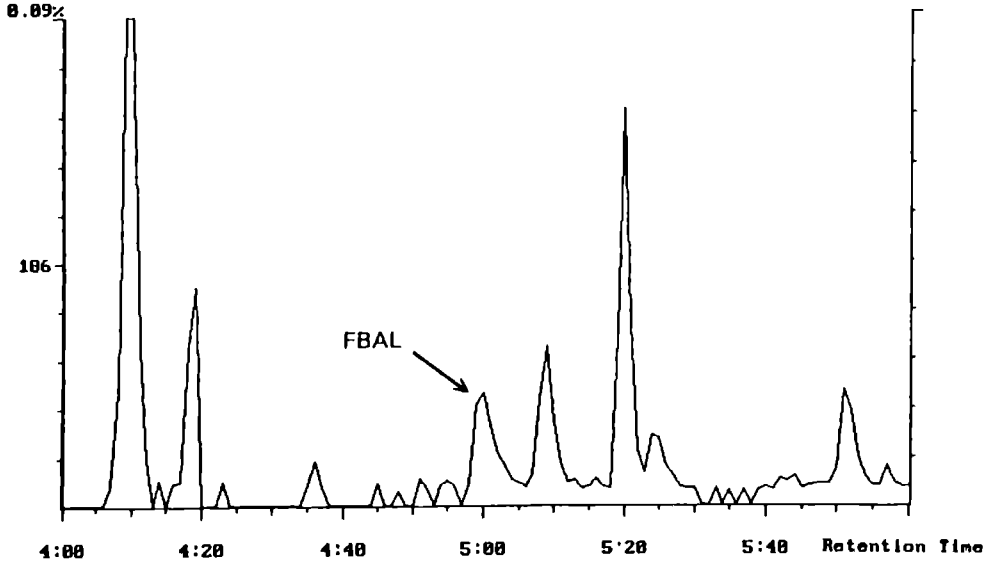
[a] nd, not detected (< 3.5 μg)
[b] ns, no sampling

Table 3. Contamination of the gloves with 5FU (μg/pair of gloves).

day	worker	activities	glove contamination
1	weigher	weighing, drug mixing, room cleaning	300
		filtration, room cleaning	22
	controller	running drug compounding process, room cleaning	64
		filtration, room cleaning	141
2	controller	coupling barrel to the production machine, removing broken vials	720[a]
		removing broken vials	ns[b]
	operator	running production machine, machine cleaning	250
		running production machine, machine cleaning	45
	packer	packing vials	44
		removing broken vials	145

[a] because of tearing three gloves were used instead of one pair
[b] the second pair of gloves was not sampled

Fig. 2. Total ion chromatogram (M = 186) of the urine sample of the weigher containing FBAL.



Discussion

This study shows that the workers were exposed to 5FU during the manufacturing process During drug compounding, the workers were mainly exposed to 5FU aerosols They were released during the manually unloading of the barrel to weigh the drug The release of 5FU was confirmed by the presence of 5FU on the filter of the mask, on the gloves, and in the air During the drug production process, the workers were potentially exposed to 5FU in solution, mainly due to broken vials The release resulted in contamination of the latex gloves. Because the latex gloves were regularly rinsed with alcohol (disinfection), the amounts detected should be regarded as an underestimation.

Before drug compounding, 5FU was detected on the floor and on several objects which were placed in the drug compounding room It is unclear what happened during cleaning, it seems as though transposition of 5FU residues had occurred

The presence of FBAL in one urine fraction of the weigher shows that 5FU was really absorbed. How the uptake did happen is not known. The presence of 5FU in the air and a non-effectively protective mask could explain the inhalatory uptake. However, dermal absorption would also be possible. In laboratory studies and in practice, it is shown that most latex gloves do not fully protect against skin uptake. Permeation was found for several antineoplastic agents including 5FU.[9,14-16] Despite the regular contact of the gloves with 5FU in solution, no FBAL was detected in the urine of the workers involved in drug production.
FBAL was excreted during the period of 10 to 13.75 hours after the beginning of weighing of 5FU. FBAL appeared later in urine of the worker when compared with a patient to whom 5FU was administered by rapid bolus intravenous injection.[5] We think that the delayed excretion of FBAL by the weigher was due to a slow skin absorption process which was out of order for the patient.

Conclusion

In the past, in situations with exposure to 5FU during occupational activities ie, drug preparation in hospital pharmacies, only the presence of 5FU in the air was established.[17,18] No methods were available to determine the uptake of 5FU by the workers. The method for the determination of FBAL in urine seems suitable for measuring the uptake of 5FU.

Appendix

Introduction

EI ionisation with the reconstructed (m/z = 186) ion chromatogram is specific for FBAL. However, specificity is increased by using MSMS. Thereto, the parent ion mass m/z = 186 is stored in the ion-trap and further split in a so-called daughter ion with m/z = 158 which is very specific for FBAL. This new application has resulted in some changes in the method which are briefly discussed below.

Gas chromatographic-mass spectrometric analysis

The samples were analysed on a Varian Saturn 4D GC/MS/MS ion-trap system with a Varian 8200 autosampler, which was controlled by a Compaq Prolinea 4/50 personal computer (software version 5.2). The initial injector temperature

was 110°C. After 1 min, the temperature was increased by 90°C/min to 280°C. After 3 min at 280°C, the temperature was decreased to the initial temperature by cooling with compressed air. The initial oven temperature was 110°C. After 1 min, the temperature was increased by 7.5°C/min to 290°C, where it remained constant for 7 min. The interface temperature was 290°C. Daughter-ion m/z = 158 was produced at an excitation amplitude of 27V (nonresonant) and an excitation RF of 300 dac. Quantification was performed on the daughter ion mass m/z = 158.

Results and discussion

MSMS mass spectra of the parent ion mass m/z = 186 of derivatized FBAL is shown in Fig. 3. High relative intensity was found for daughter ion mass m/z = 158. Fig. 4 shows the reconstructed (m/z = 186) ion chromatogram of a blank urine sample spiked with FBAL (76.2 μg/l urine) obtained by EI and a chromatogram of the daughter ion mass m/z = 158 of the same sample obtained by MSMS. It is shown that the daughter ion mass m/z = 158 obtained by MSMS further increased the specificity and the selectivity of the method. The retention time of derivatized FBAL is 5.5 min. The limit of detection is approximately 6 ng/ml urine (signal:noise ratio > 3).

Fig. 3. MSMS mass spectra of the parent ion mass m/z = 186 of derivatized FBAL.

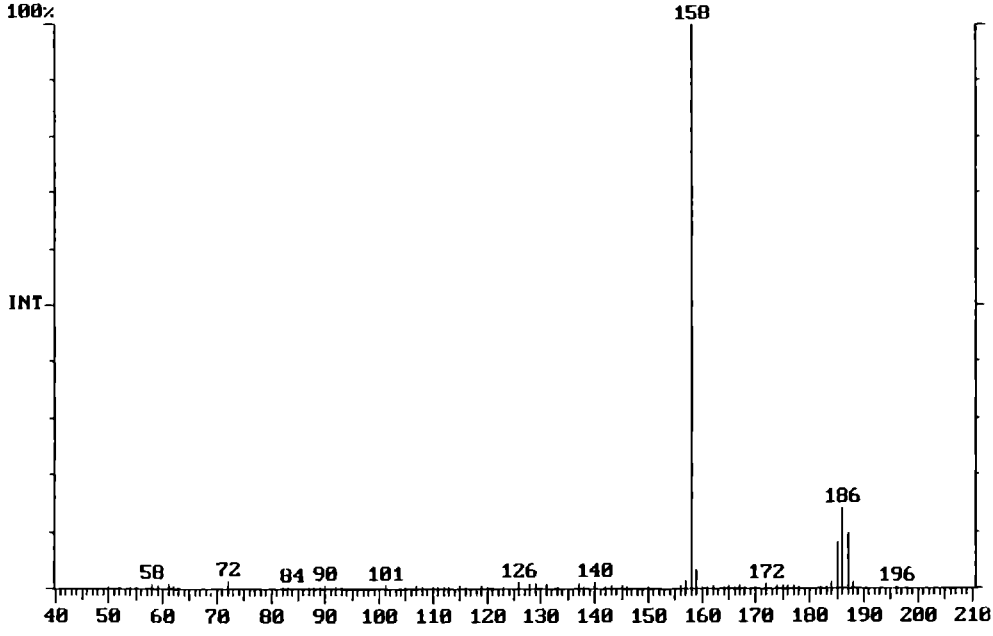
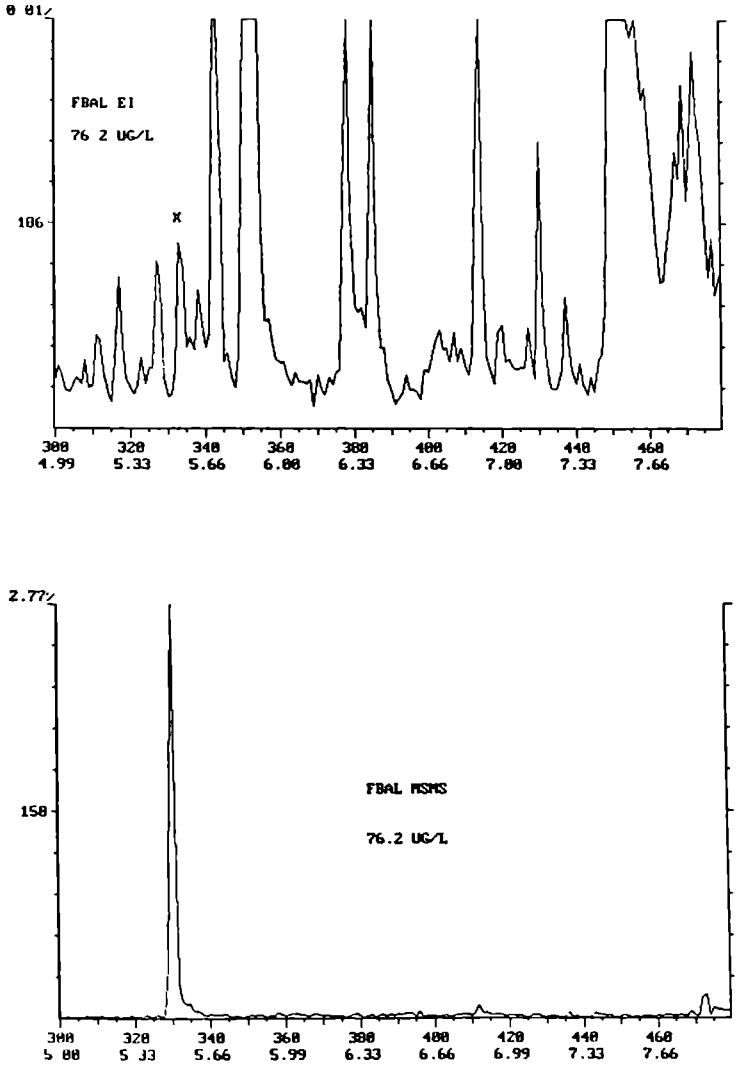


Fig. 4. *Reconstructed (m/z 186) ion chromatogram of a blank urine sample spiked with FBAL (76.2 µg/l urine) and a chromatogram of the daughter ion mass m/z = 158 of the same sample obtained by MSMS.*



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2.8

Biological and environmental monitoring of occupational exposure of pharmaceutical plant workers to methotrexate

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Summary

The exposure of 11 pharmaceutical plant workers to methotrexate (MTX) was studied. Personal air samples were taken during the different manufacturing processes: drug compounding, vial filling, and tablet preparation. The uptake of MTX was established by the determination of MTX in urine. MTX was analyzed using the fluorescence polarization immunoassay (FPIA), a method that is frequently used for monitoring serum levels in patients treated with MTX. The FPIA method was modified in such a way that MTX could be measured quickly and efficiently in air and urine samples. MTX was detected in air samples of all workers except for those involved in the vial filling process (range: 0.8-182 $\mu g/m^3$; median: 10 $\mu g/m^3$). The highest concentrations were observed for workers weighing MTX (118 and 182 $\mu g/m^3$). MTX was detected in urine samples of all workers. The mean cumulative MTX excretion over 72-96 h was 13.4 μg MTX-equivalents (range: 6.1-24 μg MTX-equivalents). A significantly lower background level of 10.2 μg MTX-equivalents was measured in urine of 30 control persons (range: 4.9-21 μg MTX-equivalents).

Introduction

Methotrexate (MTX) has been widely used in oncochemotherapy [1] and in the treatment of other nonneoplastic diseases such as psoriasis [5], rheumatoid

arthritis [6], and steroid-dependent asthma [9]. MTX primarily inhibits the enzyme dihydrofolate reductase. Consequently, DNA and RNA synthesis is decreased [1]. MTX is a teratogen and causes a variety of malformations in humans and animals [7,8]. Cytogenetic effects like sister chromatid exchanges and chromosomal aberrations have been observed in patients. MTX has been associated in case reports with a variety of subsequent neoplasms [7,8,11]. Toxic side-effects have been observed in patients [1]. In order to avoid or control these effects, serum MTX levels have been monitored.

Several assays for measuring MTX concentrations in biological fluids of patients have been reported [16]. Among these assays the enzyme-multiplied immunoassay technique (EMIT) and the fluorescence polarization immunoassay (FPIA) are the most widely used [10]. Both methods are rapid and sensitive and no interference from other matrix compounds is observed. For MTX the FPIA method is preferable for levels below 0.2 μM because of technical errors in the EMIT assay. Monitoring methods for the detection of occupational exposure to MTX are not reported in the literature.

MTX, although metabolized at all doses used in therapy, was almost completely excreted in urine [3,12]. In order to measure low levels of MTX in urine, which might be expected after occupational exposure, the FPIA method was adapted since it was primarily developed for analysis of high MTX levels in serum without sample pretreatment [10]. The FPIA method adapted for the analysis of MTX in urine was also used for the determination of MTX in air samples and was validated in a pharmaceutical plant where workers compound and produce MTX in vials and tablets.

Materials and methods

Manufacturing process and subjects

MTX was manufactured by two different processes: (1) drug compounding followed by aseptic vial filling and (2) drug compounding followed by tablet preparation. The first process took place in two different rooms (the drug compounding room and the aseptic production room). The various activities of the workers are indicated in Table 1.

Four workers were involved in drug compounding (weighing and mixing). Special protective clothes, a hair-net and sterile latex gloves were worn and the shoes were covered. Airstream helmets were only used when MTX was weighed.

Five workers were involved in aseptic vial filling. Special protective clothes, sterile latex gloves, and special boots were worn. Except for the eyes, the faces of the workers were totally covered.

Two workers were involved in drug compounding (weighing, mixing, and drying of the granulation mixture) and tablet preparation. Special protective clothes, a hair-net, sterile latex gloves, a safety mask, and special shoes were worn.
A group of 30 male and female laboratory technicians were used as nonexposed controls.

Air sampling
Personal air samples were taken during the period the workers (n = 8) were present in the different production rooms and involved in compounding (n = 4), vial filling (n = 2) and compounding/tablet preparation (n = 2) of MTX. Except for two workers involved in compounding/tablet preparation of MTX, air samples were taken from all workers once. The air samples were taken during an overall period of 6 days. Total airborne particulate matter was collected on polytetraethylene filters (diameter 37 mm, pore size 1.2 μm, Sartorius GmbH, Göttingen, Germany) with samplers (Dupont Alpha-1) that were equipped with a cone over the face (diameter 6 mm). In conjunction with a suction flow of 2.0 l/min, a suction velocity of 1.25 m/s was produced in the face entrance [15].

Urine sampling
Total urine was collected in portions during the period of about 72-96 h starting from the beginning of the working day. The amounts excreted and the excretion periods were registered. As soon as possible after collection, the urine samples were stored at -20°C until sample preparation.

Chemicals
MTX (Ledertrexate) was obtained from Lederle (Etten-Leur, The Netherlands). Reagent kits and calibrators were purchased from Abbott Diagnostics Division (Amstelveen, The Netherlands) and were ready for use. Sep-Pak C18 cartridges packed with C-18 reversed phase liquid chromatography material were obtained from Waters (Milford, Mass., USA). All other chemicals were of the highest purity obtainable.

Sample preparation and FPIA analysis of MTX
The filters of the air samples were sonicated in a 20 ml 0.03 M sodium hydroxide solution. 10 ml acetate buffer (0.05 M, pH = 4.0) was added to 10 ml sample (urine or filter extract) and the pH was set at 3.9-4.0. Sep-Pak C18 cartridges were prewashed by successive addition of 10 ml methanol and 10 ml acetate buffer. The cartidges were loaded under low pressure with sample, and washed with 10 ml acetate buffer and 10 ml water. MTX was eluted

dropwise after addition of 6 ml methanol. After evaporation of the samples under nitrogen at 45°C, 2 ml blanc human plasma was added. The samples were mixed and incubated at 37°C for 1 h. Finally, they were stored at -20°C until analysis. The recovery of MTX in the air samples was about 75%.
The samples were analyzed on a TDx analyzer (Abbott Diagnostics Division, Amstelveen, The Netherlands). The determination of MTX by the FPIA procedure was described in the Abbott TDx assays manual. In short: specific antibodies are used to isolate the desired analyte. In addition to the immunoassay reaction, FPIA incorporates three other concepts into the measurement of analytes, namely fluorescence, rotation of molecules in solution and polarized light. Methodology and applications of FPIA have been reviewed [2].
In order to evaluate the intra-assay precision of the assay, blank urine samples of five persons were spiked to a final plasma concentration of 0.28 μM MTX. Four portions of each sample were cleaned up and analyzed separately. The intra-assay precision ranged from 0% to 3.4% (mean: 2.2%). The overall recovery was 98%. A quick elution with methanol resulted in a lower response. In spiked blank urine samples (0.056 μM) stored at -20°C for 8 weeks, no loss of MTX was observed. The analytical limit of detection for MTX is 0.01 μM. Because background fluorescence levels might be present, urine samples of 30 nonexposed control persons were analyzed. The mean background level found corresponded with 10.2 μg MTX-equivalents (range: 4.9-21 MTX-equivalents). In air samples no background levels were observed.

Statistical analysis

Statistical analysis was carried out on a personal computer using InStat 1.1 as software package. A P value < 0.05 was considered to be of statistical significance.

Results

Air samples

MTX was detected in air samples of all workers except for those involved in the vial filling process (Table 1). The highest air concentrations were observed for the workers who compound MTX, among whom workers 2 and 3 weighed MTX and were highly exposed. Lower air concentrations were found for the workers involved in the drug compounding/tablet preparation process. The limit of detection was about 0.07 $\mu g/m^3$.

Table 1. Personal air sampling of MTX[a].

process	worker	sampling time (min)	MTX ($\mu g/m^3$)
drug compounding	1	210	0.8
	2	306	182
	3	135	117
	4	205	46
vial filling	5	ns[b]	
	6	ns	
	7	ns	
	8	180	nd[c]
	9	180	nd
drug compounding/tablet preparation	10	47	13
		150	1 6
		35	10
	11	90	5.4
		95	2.6

[a] on blank filters MTX was not detected (<0.03 μg)
[b] ns, no sampling
[c] nd, not detected (<0.07 $\mu g/m^3$)

Urine samples

The results of the urinary MTX excretion of the workers are presented in Table 2. Because background fluorescence levels might be present in the urine samples, the amounts of MTX are expressed in MTX-equivalents. For the workers involved in drug compounding, the cumulative excretion over 72 h ranged from 6.4-18 μg MTX-equivalents. In urine samples of all workers involved in the vial filling process, the cumulative MTX excretion over 72 h ranged from 8.7-21 μg MTX-equivalents. The cumulative 96-h excretion was 6.1 and 24 μg MTX-equivalents for the two workers involved in compounding/tablet preparation. The mean urinary MTX excretion of the total group was 13.4 μg MTX-equivalents. This was significantly higher than the urinary MTX excretion of 10.2 μg MTX-equivalents of 30 control persons ($P = 0.0078$, two-tailed t-test). When the three groups were considered separately, significantly higher levels of MTX-equivalents were only found for the group of four workers involved in drug compounding ($P = 0.0207$, two-tailed t-test).

Table 2. Urinary excretion of MTX.

process	worker	MTX-equivalents (μg)
drug compounding	1	7.8
	2	6.4
	3	13
	4	18
vial filling	5	21
	6	13
	7	20
	8	8.7
	9	9.5
drug compounding/tablet preparation	10	24
	11	6.1

Discussion

The results of the analysis of the air samples show release of MTX during the drug compounding and the drug compounding/tablet preparation process. The highest air concentrations were observed for the workers weighing MTX. Lower air concentrations were detected for the workers involved in drug compounding/tablet preparation. During both processes MTX was used as a powder. After dissolution in water, no detectable amounts of MTX were observed in the air (vial filling process). It was somewhat surprising that only low amounts of MTX were found during the drug compounding/tablet preparation process because of the expected release of MTX during sifting of the granulation mixture.

Urine analysis shows that, although protective measures were taken, the uptake of MTX was significantly higher for the total group of workers when compared with the nonexposed controls. When the three groups were considered separately, significantly higher MTX levels were only found for the group of workers involved in drug compounding. It is striking that these workers also showed the highest air concentrations of MTX.

In addition to the inhalation route, dermal and oral uptake (finger-shunt effect) cannot be excluded. Although the workers used protective latex gloves, it is known that MTX permeates some of such gloves [4,13,14]. Also the use of

several additives like sodium hydroxide and alcohol for cleaning purposes could influence permeation and penetration resulting in uptake of MTX by skin. In summary, several factors might influence the excretion of MTX observed for the workers. The data on excretion of MTX do not permit the development of a kinetic model in the case of occupational exposure to this drug. This could be helpfull in the development of a simple biomonitoring method. Nevertheless, the presented FPIA method may be a feasible tool for the detection of MTX in air and urine samples of workers occupationally exposed to MTX, but this has to be proven in a larger study.

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Chapter 3

Biological effects in workers exposed to antineoplastic agents

3.1

Urinary cyclophosphamide excretion and chromosomal aberrations in peripheral blood lymphocytes after occupational exposure to antineoplastic agents

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Abstract

In this study we have compared the results of a method for the detection of cyclophosphamide in urine and the results of analysis of chromosomal aberrations in peripheral blood lymphocytes of four groups of subjects with various exposure statuses. These groups are 17 Dutch and 11 Czech exposed workers (mainly hospital nurses and pharmacy technicians) handling antineoplastic agents and 35 Dutch and 23 Czech controls (nurses, medical doctors, pharmacy and lab technicians) not handling these drugs. The groups were subdivided into smokers and non-smokers because of a confounding effect of smoking.

Within the Dutch groups, the percentage of aberrant cells and the number of breaks per cell were increased for smokers compared to non-smokers. The percentage of aberrant cells was increased in Dutch exposed workers in comparison with Dutch control workers. Within the Czech groups the percentage of aberrant cells and the number of breaks per cell were increased in exposed workers in comparison with control workers. However, both Dutch and Czech smokers mainly contributed to the increase. The results suggest an additive effect of exposure and smoking in the Dutch subjects and a more than additive effect in the Czech subjects.

In urine samples of three out of 11 Dutch exposed workers cyclophosphamide was found in a range of 0.1-0.5 μg/24 h. Higher levels were detected in the

urine of eight out of 11 Czech exposed workers, a range of 0.1-2.9 μg/24 h. No correlation was observed between the amounts of cyclophosphamide excreted in urine on the one hand and the percentage of aberrant cells and the number of breaks per cell on the other hand.
The present study is the first study showing that hospital workers having an increase in chromosome aberrations related to their work are exposed to at least one antineoplastic agent.

Introduction

Many antineoplastic agents are carcinogenic, mutagenic and teratogenic in laboratory animals (IARC, 1981). Some antineoplastic agents are human carcinogens since for instance secondary tumors were found in patients treated with these drugs (Schmähl and Kaldor, 1986). It is not known whether persons occupationally involved in the synthesis, formulation, preparation, and administration of these drugs are at risk. Therefore, special guidelines and safety precautions were introduced to protect workers from contact with these toxic compounds (ASHP, 1990; Integrale Kankercentra, 1992).
Several biomonitoring methods for the detection of occupational exposure to antineoplastic agents have been developed and validated. Methods like the determination of mutagenicity and thioethers in urine and cytogenetic methods like the analysis of chromosomal aberrations (CA), sister-chromatid exchanges and micronuclei in peripheral blood lymphocytes have been used frequently (for reviews see Sorsa et al., 1985; Kaijser et al., 1990). Methods for the detection of specific antineoplastic agents or their metabolites in urine of exposed workers have been developed for cyclophosphamide (CP) (Hirst et al., 1984; Evelo et al., 1986; Sessink et al., 1992, 1993a,b, 1994b), cis-platinum (Venitt et al., 1984), ifosfamide (Sessink et al., 1992b) and 5-fluorouracil (Sessink et al., 1994a).
In this study we have compared the results of a method for the detection of CP in urine with the results of analysis of CA in peripheral blood lymphocytes for four groups of subjects with various exposure statuses. These groups were Dutch and Czech exposed workers handling antineoplastic agents and Dutch and Czech controls not handling these drugs. The aim of this study was to discuss the usefulness of the two methods with respect to the possibility of biomonitoring occupational exposure to antineoplastic agents.

Materials and methods

Subjects

Four groups were studied:

-17 Dutch hospital nurses and pharmacy technicians involved in the preparation and administration of antineoplastic agents;
-35 Dutch control pharmacy technicians working in regular pharmacies and not handling antineoplastic agents;
-11 Czech cleaning women, nurses and a lab technician involved in the administration and preparation of antineoplastic agents, all working in the same department of a hospital;
-23 Czech control nurses, medical doctors, lab technicians and a cleaning woman not handling antineoplastic agents.

Sex, age, profession, exposure period, smoking habits, alcohol use, diseases, medicine use, inoculation and exposure to chemicals and X-rays were investigated by means of a questionnaire. The characteristics of the groups are shown in Table 1. Most of the workers handling antineoplastic agents wore gloves, masks and special clothes. The drugs were prepared in laminar down-flow hoods. The amounts of the drugs prepared and administered and the periods of handling were registered. In addition to CP, ifosfamide, cis-platinum, 5-fluorouracil, methotrexate, cytarabine, doxorubicin, etoposide, vinblastine and vincristine were most frequently prepared and administered.

Chromosome aberrations in blood lymphocytes

Peripheral blood samples were taken by venous puncture. The blood was collected in vacuum tubes containing heparin. The blood samples were coded, cooled at 4-10°C and immediately sent to the laboratory in Prague for the determination of CA. Sample preparation has been described elsewhere (Pohlová et al., 1986). The culture was incubated at 37°C for 50 h. One hundred metaphases were examined for each worker. Chromosome and chromatid breaks and exchanges (dicentrics, rings, translocations) were counted and the percentage of aberrant cells (%AB.C) and the number of breaks per cell (B/C) were calculated.

Table 1. Distribution by sex, age, exposure period and smoking habits of control and exposed workers.

	n[a]	sex		age		years of exposure		smokers		
		male	female	mean	range	mean	range	n[b]	number of cigarettes/day	
									mean	range
dutch control	35	0	35	29	21-54			7	14	8-15
dutch exposed	17	0	17	35	25-50	7	4-14	2	14	12-15
czech control	23	3	20	44	27-59			4	17	13-20
czech exposed	11	0	11	39	28-53	13	3-25	6	10	10

[a] n, number of subjects (smokers and non-smokers)
[b] n, number of smokers

Cyclophosphamide in urine

Total 24-h urine was collected in portions starting from the end of the preparation/administration of the drugs No urine samples were taken from the Dutch controls because previous studies had demonstrated that CP could not be detected in Dutch persons not exposed to antineoplastic agents. From the Czech control group urine samples were taken starting at the end of the working day. The amounts of urine excreted and the excretion periods were registered. The urine samples were coded and stored at -20°C until sample preparation. After preparation by liquid-liquid extraction with diethylether andderivatization with trifluoracetic anhydride, the coded urine samples were analyzed using gas chromatography-mass spectrometry. The limit of detection was about 0 25 ng/ml urine. Details about sample preparation and analysis have been described elsewhere (Sessink et al., 1992b, 1993b).

Statistical analysis

Statistical analysis was carried out using SAS software version 6.07. Two-way analysis of variance (SAS procedure GLM) was used to study influence of exposure and smoking. Only P-values equal to or below 0.05 were considered to be significant.

Results

Chromosome aberrations in blood lymphocytes

The results of the analysis of CA in the peripheral blood samples are presented in Table 2. Only comparisons within the Dutch and within the Czech groups were taken into account because it was expected that more or less country-specific confounders could introduce differences between the groups. The groups were subdivided into smokers and non-smokers because confounding due to smoking was also expected.

Within the Dutch groups, the percentage of aberrant cells (%AB C) and the number of breaks per cell (B/C) were increased for smokers compared to non-smokers. The %AB.C was increased in Dutch exposed workers in comparison with Dutch control workers. Within the Czech groups the %AB.C and the number of B/C were increased in exposed workers in comparison with control workers. However, both Dutch and Czech smokers mainly contributed to the increase. Dutch and Czech exposed smokers showed the highest %AB.C and number of B/C in comparison with their corresponding exposed and control non-smokers and smoking controls. The results suggest an additive effect of exposure and smoking in the Dutch subjects and a more than additive effect in the Czech subjects.

Table 2. Chromosome aberrations (mean ± S.D.) in peripheral blood lymphocytes of control and exposed workers.

		non-smokers			smokers			all		
		n[a]	%AB.C[b]	B/C[c]	n	%AB C	B/C	n	%AB.C	B/C
dutch	control	28	1.9 ± 1.4	0.020 ± 0.014	7	2.7 ± 2.1	0.039 ± 0.032	35	2.1 ± 1.6[d]	0.024 ± 0.020
	exposed	15	2.3 ± 1.1	0.027 ± 0.014	2	5.0 ± 0.0	0.050 ± 0	17	2.6 ± 1.4[d]	0.030 ± 0.015
	all	43	2.0 ± 1.3[f]	0.023 ± 0.014[g]	9	3.2 ± 2.1[f]	0.042 ± 0.029[g]	52	2.3 ± 1.5	0.026 ± 0.019
czech[h]	control	19	1.9 ± 1.3	0.022 ± 0.014	4	1.0 ± 0.8	0 010 ± 0.008	23	1.8 ± 1.3[e]	0.020 ± 0.013[i]
	exposed	5	1.8 ± 2.0	0.026 ± 0.030	6	3.5 ± 0.8	0.040 ± 0.009	11	2.7 ± 1.7[e]	0.034 ± 0.021[i]
	all	24	1.9 ± 1.4	0.023 ± 0.017	10	2.5 ± 1.5	0.028 ± 0.018	34	2.1 ± 1.5	0 024 ± 0.017

[a] n, number of subjects
[b] %AB.C, percentage of aberrant cells (breaks and exchanges)
[c] B/C, number of breaks per cell
[d e] significant difference between exposed and control (P = 0.04)
[f g] significant difference between smokers and non-smokers (P = 0.007)
[h] significant more than additive effect between exposure and smoking (%AB.C: P = 0.02; B/C: P = 0.05)
[i] significant difference between exposed and control (P = 0.01)

Cyclophosphamide in urine

In the Dutch exposed group 79 urine samples were analyzed from 11 out of 17 workers (Sessink et al., 1992b). In three urine samples from three workers CP was detected. The amounts were 0.1 and 0.5 μg/24 h (non-smokers) and 0.3 μg/24 h (smoker).

The results of the Czech exposed groups are presented in Table 3. Sixty-eight urine samples of 11 workers were analyzed. In 35 samples of eight workers CP was detected in a range of 0.1-2.9 μg/24 h. CP was found in the urine of workers who were (n = 7) and who were not (n = 2) directly involved in the preparation or administration of CP. In the urine of smokers (n = 3) and non-smokers (n = 5) CP was present. Even in the urine of a cleaning woman involved in the clearing of cytostatic waste after the administration of these drugs, CP was detected. In the Czech control group 147 urine samples were analyzed. In one sample obtained from a cleaning woman 0.3 μg CP was found. No correlation was observed between on the one hand the amounts of CP excreted in urine and on the other hand the %AB.C and the number of B/C in the exposed workers.

Table 3. Cumulative CP excretion (μg/24 h) of Czech exposed workers in relation to profession, smoking habits and handling antineoplastic agents.

worker/profession	handling antineoplastic agents (±CP)[a]	smoking habits[b]	CP in 24 h urine
nurse	administration (-CP)	10	nd[c]
nurse	preparation (-CP)	0	0.3
nurse	preparation and administration (+CP)	10	nd
nurse	preparation and administration (+CP)	10	1.4
nurse	preparation and administration (+CP)	10	2.9
nurse	preparation and administration (+CP)	0	0.2
nurse	preparation and administration (+CP)	0	0.9
nurse	preparation and administration (+CP)	0	2.4
lab technician	preparation and administration (+CP)	0	0.5
cleaning woman	waste clearing after administration (+CP)	10	0.1
cleaning woman	waste clearing after administration (+CP)	10	nd

[a] (-CP), without CP; (+CP), including CP
[b] number of cigarettes/day
[c] nd, not detected

Discussion

Cytogenetic methods like the analysis of CA in blood lymphocytes have been frequently used for the biological (effect) monitoring of occupational exposure to antineoplastic agents. Results of these studies were different in nature. Sometimes an increase in CA was found in exposed workers compared to controls (Waskvik et al., 1981; Nikula et al., 1984; Pohlová et al., 1986; Oestreicher et al., 1990; Milkovic-Kraus and Horvat, 1991; Grummt et al., 1993). More frequently, no differences between exposed and control workers were observed (Kolmodin-Hedman et al., 1983; Stiller et al., 1983; Stucker et al., 1986; Benhamou et al., 1988; Sorsa et al., 1988; Krepinsky et al., 1990; Sarto et al., 1990; Cooke et al., 1991). The different results might be explained by variable levels of exposure, the epidemiological approach and technical problems in sample preparation and analysis (Grummt et al., 1993; Oestreicher et al., 1990).

The analysis of CA should be considered only at a group level to indicate exposure to genotoxic agents like antineoplastic agents. Matched control groups were used because of possible confounders like age, sex, smoking and exposure to X-rays and chemicals. When no matched groups were used, the groups were subdivided depending on the confounder in question. In some studies this was done for smoking. However, no correlation was found between smoking habits and CA (Waskvik et al., 1981; Pohlová et al., 1986; Grummt et al., 1993) or not enough smokers participated in the study (Benhamou et al., 1988). Finally, none of these studies gave exposure data of specific antineoplastic agents.

Our results indicate that the influence of exposure to antineoplastic agents was only observed within the Czech group when exposed workers were compared to controls (Table 2). However, when the groups were subdivided into smokers and non-smokers, an influence of exposure on the %AB.C and the number of B/C was only found for smokers. Within the Dutch groups, the %AB.C and the number of B/C were increased for smokers compared to non-smokers and for exposed workers compared to control workers. About the mechanism one may speculate. In the past, it has also been described that smoking had a synergistic action on the appearance of mutagens in the urine after exposure to antineoplastic agents (Bos et al., 1982; Breed et al., 1986). In both studies a significant increase in biologically active mutagenic metabolites (no bioactivation was needed) was found in urine of smoking nurses handling antineoplastic agents in comparison with smoking controls and non-smoking nurses. Two possible explanations were given. First, due to smoking, induction of enzymes of the mono-oxygenase system involved in the biotransformation of CP could be responsible, and, second, differences in exposure and working hygiene (a possible finger-shunt effect in smokers resulting in an increased oral uptake) might play a role.

Our results of the analysis of CP in urine show that when the Czech workers were handling CP (and other antineoplastic agents) this particular compound was absorbed. No confounding due to smoking was observed, because in urine of both, smokers and non-smokers, CP was detected. The presence of CP in one urine sample of a control cleaning woman is remarkable. How this might have happened is unclear.

CP belongs to the most abundantly used genotoxic carcinogenic antineoplastic agents in The Netherlands. Our results suggest that urinary levels of CP up to 2.9 μg/24 h may result in biological effects like CA in the long run. Since CA are considered to be indicators of an increased genetic risk, these urinary CP concentrations may not only show absorption of this particular antineoplastic drug but may also indicate a serious risk at these levels after some period of working, especially for smokers.

The determination of CP in urine can be used for the detection of acute exposure to CP while the analysis of CA can be used for the detection of long-term exposure to antineoplastic agents because lymphocytes carrying aberrations may persist for months and maybe years. The analysis of CA alone is not a reliable indicator of occupational exposure to genotoxic antineoplastic agents because many factors do influence the results of this method. Measurement of CA must be considered a marker of an early biological response. To monitor exposure the use of a sensitive analytical method like the determination of CP in urine seems very accurate. The detection limit of CP using gas chromatography-mass spectrometry is about 25 pg which makes it possible to detect less than nanograms of CP per ml urine (Sessink et al., 1993b). Since procedures for preparation and administration are almost the same for all antineoplastic agents, the combination of the frequent use of CP in oncochemotherapy and the availabilty of a sensitive method for the determination of CP in urine makes the method suitable for monitoring exposure to CP in particular and possibly to antineoplastic agents in general (signal function).

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3.2

Occupational exposure to antineoplastic agents and parameters for renal dysfunction

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Abstract

To study the nephrotoxic effects of occupational exposure to antineoplastic agents, the early renal effect parameters retinol-binding protein (RBP) and albumin (ALB) were determined in the urine of 11 hospital workers involved in the preparation and administration of antineoplastic agents and in 23 hospital workers not involved in drug handling, who served as nonexposed controls. No significant difference was found between the exposed group and the nonexposed control group with respect to the early renal effect parameters RBP and ALB. Although it was demonstrated that the hospital workers were exposed to cyclophosphamide (CP) and probably other antineoplastic agents, the results of the present study show that these exposure levels did not cause nephrotoxic effects.

Introduction

Urinary proteins such as $ß_2$-microglobulin, albumin (ALB), retinol-binding protein (RBP), and the urinary activity of enzymes such as alanine aminopeptidase, ß-galactosidase and N-acetyl-ß-D-glucosaminidase are early renal effect parameters that have proven useful for the early detection of nephrotoxicity. These parameters are widely used in occupational medicine for the detection of nephrotoxic effects in workers exposed to chemicals [16,35].

They are also applied clinically to control nephrotoxic side-effects of drugs such as aminoglycosides [3] and antineoplastic agents such as cisplatin [2,3,6,8,9,12,14,15,19,30,32,33,35].
In a number of studies we have demonstrated that workers involved in the production, preparation and administration of antineoplastic agents are exposed to these drugs [5,22-29]. The uptake was assessed by the determination of the parent drug or metabolite in the urine of the workers. Large differences were found in urinary excretion of cyclophosphamide (CP) between several groups, suggesting variable exposure levels [5,23,24,26,28]. The question is whether these exposure levels will cause nephrotoxic effects in the workers.
Recently, we presented the results of a study in which the urinary CP excretion was determined together with the analysis of chromosomal aberrations in peripheral blood lymphocytes of Dutch and Czech exposed and nonexposed hospital workers [26]. It was shown that chromosomal aberration frequencies were higher in the groups of exposed hospital workers than in their nonexposed controls. The highest amounts of CP were found in the urine samples of 11 Czech exposed hospital workers (0.1-2.9 μg/24 h). For a worst-case approach, this group and their corresponding nonexposed control group ($n = 23$) were selected for monitoring of early renal effects. Urinary ALB and RBP were selected as the most appropriate parameters [35].

Materials and methods

Subjects

Two groups were studied:
11 nurses, cleaning women and a lab technician involved in the preparation and administration of antineoplastic agents, all working in the same department of a hospital;
23 control nurses, medical doctors, lab technicians and a cleaning woman not handling antineoplastic agents.
Sex, age, profession, exposure period, smoking habits, alcohol use, illnesses, medicine use, inoculation and exposure to chemicals and X-rays were investigated by means of a questionnaire. From this, it appearred that the two groups were rather comparable. There were no indications that persons involved in this study could bring about a confounding effect. Some characteristics of the groups are shown in Table 1. Most of the workers handling antineoplastic agents used gloves, masks and special clothes. The agents were prepared in laminar down-flow cabinets. The amounts of the agents prepared and administered and the periods of handling were registered. CP, isophosphamide, cisplatin, 5-fluorouracil, methotrexate, cytarabine, doxorubicin, etoposide, vinblastine and vincristine were most frequently prepared and administered.

Urine sampling and analysis

Total 24-h urine was collected in portions starting at the end of the preparation/administration of the drugs. The same urine samples were used as in a previous study for the determination of CP [26]. Morning urine samples were used for analysis of RBP and ALB. Both proteins were determined by latex immunoassay [11] and were adjusted for dilution with the urinary creatinine (CREAT-U) concentration [35]. All parameters were analyzed within one run. The mean concentration and the duplicate precision (between brackets) of the determinations of the early renal effect parameters were: RBP 126.4 μg/l (9.6%); ALB 5.9 mg/l (6.4%).

Statistical analysis

The nonnormal distributions of the renal effect parameters ALB and RBP were transformed logarithmically. Differences in both parameters between the exposed group and the nonexposed control group were tested with Student's *t*-test. Correlations were quantified with the Pearson correlation coefficient, *r*. *P*-values (two-tailed) below 0.05 were considered to be of statistical significance. The statistical analysis were carried out on a personal computer using the InStat 1.1 software package.

Results

The mean concentrations of the renal effect parameters ALB and RBP in the exposed group and the nonexposed control group are presented in Table 2. No significant difference was observed between the two groups in the concentrations of these parameters. No correlation was found between the number of years of exposure and the urinary ALB ($r=0.31$; $P=0.50$) or RBP concentration ($r=0.25$; $P=0.59$). In addition, ALB and RBP concentrations were not related ($r=0.33$; $P=0.33$). The effect of smoking was also investigated. To this end, the exposed group and the nonexposed control group were subdivided into smokers and nonsmokers. Two-way analysis of variance (SAS General Linear Models procedure, software version 6.07) was used to study the influence of smoking and exposure. No significant effect of smoking was found upon the urinary ALB and RBP excretion.

Table 1. *Distribution by sex, age, exposure period and smoking habits in the exposed and nonexposed group of Czech hospital workers.*

	n[a]	sex		age		years of exposure		smokers		
		male	female	mean	range	mean	range	n[b]	number of cigarettes/day	
									mean	range
exposed	11	0	11	39	28-53	13	3-25	6	10	10
nonexposed	23	3	20	44	27-59			4	17	13-20

[a] number of subjects (smokers and nonsmokers)
[b] number of smokers

Table 2. Mean concentrations of the renal effect parameters albumine (ALB) and retinol-binding protein (RBP) in the exposed and nonexposed group of Czech hospital workers.

parameter	geometric means (range)		P^a
	exposed (n = 11)	nonexposed (n = 23)	
ALB (mg/mol CREAT-U)	0.44 (0.09-2.33)	0.30 (0.03-0.95)	0.27
RBP (μg/mol CREAT-U)	7.9 (3.0-18.9)	8.8 (1.2-28.0)	0.71

[a] two-tailed *t*-test

Discussion

Urinary renal effect parameters are widely used for the detection of early renal dysfunction in workers exposed to nephrotoxic chemicals such as organic solvents, heavy metals and silica [16,20]. In patients treated with some antineoplastic agents, nephrotoxic side-effects are observed [2,3,6,8,9,12,14,15,19,30,32, 33,35]. The question is whether nephrotoxic effects occur in hospital workers who are occupationally exposed to antineoplastic agents. Up to now, no such data have been published.

Several studies have shown that groups of hospital workers are exposed to antineoplastic agents [4,5,13,17,23,24,26-29,34]. This was found by measurement of the parent compound or metabolites in the urine of the workers. Pharmacokinetic studies have shown that large amounts of antineoplastic agents administered to patients are excreted unchanged in their urine [1,7,10,21,31]. These levels are much higher than those found in the urine of hospital workers involved in the preparation and administration of these agents. However, it remains unclear to what extent chronic exposure to low amounts of antineoplastic agents will cause adverse health effects. In a previous study, we showed that occupational exposure to these drugs may cause genetic effects [26]. It appeared that chromosomal aberration frequencies in peripheral blood lymphocytes of hospital workers handling antineoplastic agents were increased over those in their controls. The highest increase was observed in the exposed smokers, suggesting an additive effect of exposure and smoking. Therefore, it is not impossible that these exposure levels may cause other health effects, such as nephrotoxicity. In order to detect possible nephrotoxic effects at an early stage, the excretion of urinary

ALB and RBP was determined.
Recently, it was found in a large study involving 5670 people that urinary ALB concentrations were significantly higher in smokers than in nonsmokers [18]. Hence, it is reasonable to assume that smoking is a possible confounder. However, in our study no significant effect of smoking on urinary ALB and RBP excretion was found. One explanation could be that our study was conducted in 34 hospital workers.
In conclusion, our results show no difference in urinary ALB and RBP excretion between the exposed group and the nonexposed control group of hospital workers. However, it should be noted that in our (pilot)study rather small groups were monitored. No increase in urinary RBP excretion indicates no tubular dysfunction. No increase in urinary ALB excretion and urinary RBP excretion indicates no tubular and no glomerular renal dysfunction. Although a previous study demonstrated that the hospital workers handling antineoplastic agents were exposed to one of these drugs, the results of the present study indicate that these exposure levels do not result in nephrotoxic effects.

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Chapter 4

Toxicokinetic studies on cyclophosphamide (uptake, biotransformation, and excretion)

4.1

Urinary cyclophosphamide excretion in rats after intratracheal, dermal, oral and intravenous administration of cyclophosphamide

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Summary

The urinary excretion of unmetabolized cyclophosphamide (CP) was studied in rats after intratracheal, dermal, oral and intravenous administration. Rats were given two doses of 1 mg kg^{-1} CP 48 h apart and urine was collected for 96 h after the first treatment.
With the help of a phosphor-specific filter in a flame photometer attached to a gas chromatograph, low levels of CP were determined after derivatization with trifluoroacetic anhydride.
Cumulative excretion as a percentage of dose ranged from 4.0 to 6.9 after the first dose and 2.7 to 5.5 after the second dose. The highest rate of excretion after the second administration was observed in rats treated intratracheally, while cumulative excretion was higher (6.9%) after the first than after the second (2.7%) intravenous treatment. The most prolonged excretion was observed after dermal application.

Introduction

During the last decade, much attention has been paid to the determination of occupational exposure to cytostatic drugs in hospitals and industry.[1,2] Most so-called biomonitoring methods are non-selective, which means that the parameters measured can be influenced by more than one agent (e.g. urinary mutagenicity, chromosome aberrations and sister chromatid exchanges in blood lymphocytes).[3-9]

To follow the uptake of cytostatic drugs at low-level applications requires the development of selective and sensitive methods.[10,11] This paper deals with the estimation of cyclophosphamide (CP), one of the most applied parenteral cytostatic drugs in The Netherlands during 1986-1988.[12]
Inhalation and absorption through the skin are expected to be the most important routes of occupational exposure to cytostatic drugs.[11,13,14] In this study, the excretion of unmetabolized CP in rat urine was investigated after intratracheal instillation and dermal application. We have investigated whether the initial exposure can influence the excretion rate of CP given 48 h later. The results found are compared with the results obtained after oral and intravenous administration.

Experimental

Chemicals

CP (endoxan; purity >97%) and isophosphamide (IP) (holoxan; purity >97%) were purchased from ASTA-Pharma (Bielefeld, FRG). Trifluoroacetic anhydride (TFAA; purity >98%) was obtained from Johnson Mattey GmbH (Karlsruhe, FRG) and ethyl acetate (purity >99.8%) and n-hexane (purity >95%) were bought by Labscan Limited (Dublin, Ireland). Glycerol (purity ±87%) and toluene (purity >99.5%) were obtained from Merck (Darmstadt, FRG).

Animals

Five groups of three male CPB:WU (Wistar) rats weighing 180-220 g were housed individually in stainless-steel metabolism cages for 5 days before treatment with CP. Rats had a conventional microbiological status and had free access to water and RMH-TM pellets (Hope Farms BV, Woerden, The Netherlands). Urine samples and faeces were collected seperately.
CP was administered to ether-anaesthetized rats at a dose level of 1 mg kg^{-1} body weight. For oral, intravenous (penis vene) and intratracheal treatment, a saline solution was used. For dermal exposure, a glycerol suspension was applied on ca. 2 cm^2 of the close-shaven skin of the neck. Control rats received no CP. Fourty-eight hours after the first administration, rats received a second treatment with the same dose. Eight-hour urine samples were collected on ice-water for 4 days after the first administration, adjusted to 10 ml with distilled water, shaken and stored at -20°C until sample preparation. Blank urine samples spiked with CP were prepared immediately after the first and the second treatment, and were used as references.

Sample preparation

One millilitre of Tris buffer (0.2 M; pH 4.5) and 100 μl of internal standard (5 μg IP ml^{-1} distilled water) were added to 1 ml of urine sample (in duplicate).

The samples were extracted twice with 5 ml of ethyl acetate and the ethyl acetate layers were combined in conical tubes with screw caps. Samples were dried under nitrogen at 40°C and shaken with 50 µl of ethyl acetate until the residue was totally dissolved. TFAA (50 µl) was added and, after shaking, the tubes were closed for derivatization for 20 min at 70°C.[10] At room temperature the samples were supplemented with 500 µl of saturated NaCl solution and extracted twice with *n*-hexane. Hexane extracts were pooled and dried under nitrogen at 40°C. Next, 50 µl of toluene were added. After sonification, the vials were stored at -20°C until analysis.

GC analysis

The gas chromatograph (Carlo Erba 5300) was connected to a flame photometer detector (control module type SSD 250; Carlo Erba) with a phosphor-specific emission filter (wavelength 526 nm). For peak integration (peak area), a Spectra Physics SP 4290 integrator was used.

The splitless injection mode was used (splitless time 30 s; injector temperature 250°C; 1 µl injection). Carrier and make-up gas flows (He) were 1.0 and 25 ml min^{-1}, respectively. A 10 m SE-54-DF fused-silica column (Permabond Macherey-Nagel, Düren, FRG) was used, with 0.32 mm internal diameter and 0.5 µm film thickness. The detection temperature was 250°C and the excitation dial was set on 7.0. Air and hydrogen flows were 110 and 100 ml min^{-1}, respectively. The initial temperature was 75°C. After 1 min, the temperature was raised to 250°C (20°C min^{-1}). After 5 min at 250°C, the temperature was raised at the same rate to 275°C, where it remained constant for 10 min.

Quantitation of the trifluoroacetyl derivatives was carried out by reference to calibration curves constructed from the analysis of reference urine samples.

Results

A typical chromatogram of a urinary sample containing 10 ng of IP and 1 ng of CP is shown in Fig. 1. The TFAA derivatives of IP and CP were separated completely and no interference with other compounds was found. Retention times of derivatized IP and CP were ca. 12.6 and 13.9 min, respectively. All reference urine curves used in this study were linear (range 4-2000 ng ml^{-1} urine). The limit of detection (signal-to-noise ratio of 3:1) was ±6 ng ml^{-1} urine. All urine samples were prepared in duplicate and gave a coefficient of variation of 7.9 % (n = 59).

Figure 2 shows the excretion of unmetabolized CP in urine of rats as a percentage of the dose after intratracheal, dermal, oral and intravenous administration.

No CP was present in urine samples of rats 24 h after intravenous treatment,

32 h after intratracheal and oral treatment and 40 h after dermal application. In control urine samples, no CP was found.
Intravenous injection (first treatment) and intratracheal instillation (second treatment) gave the highest excretion rate and dermal application gave the lowest (both treatments).
Table 1 shows the cumulative CP excretion in the first 32 h after treatment. The percentage excretion declined in the following order: intravenous injection, dermal, intratracheal and oral treatment. The order of excretion after the second dose was: intratracheal, dermal, oral and intravenous treatment.

Fig. 1. Gas chromatograph-flame photometer detector chromatogram of a rat urine sample containing 10 ng of IP and 1 ng of CP.

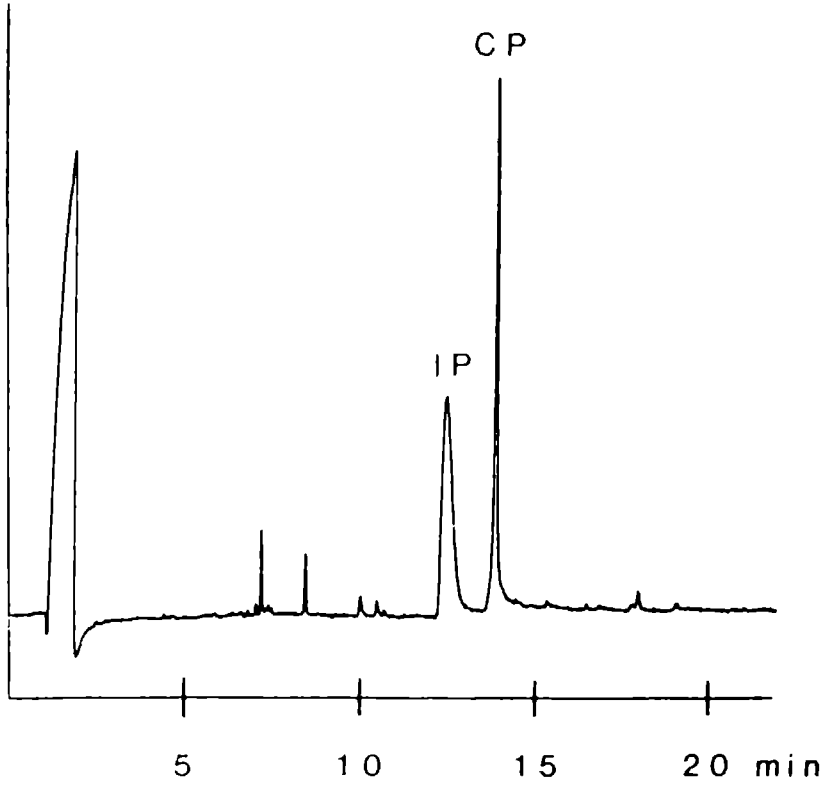

Fig. 2. Excretion in 8-h periods of unmetabolized CP in rat urine as a percentage of the dose (1 mg kg^{-1} body weight) after intratracheal (A), dermal (B), oral (C) and intravenous (D) administration (mean of three rats ± S.D.). The second treatment was 48 h after the first treatment. Determinations were performed in duplicate.

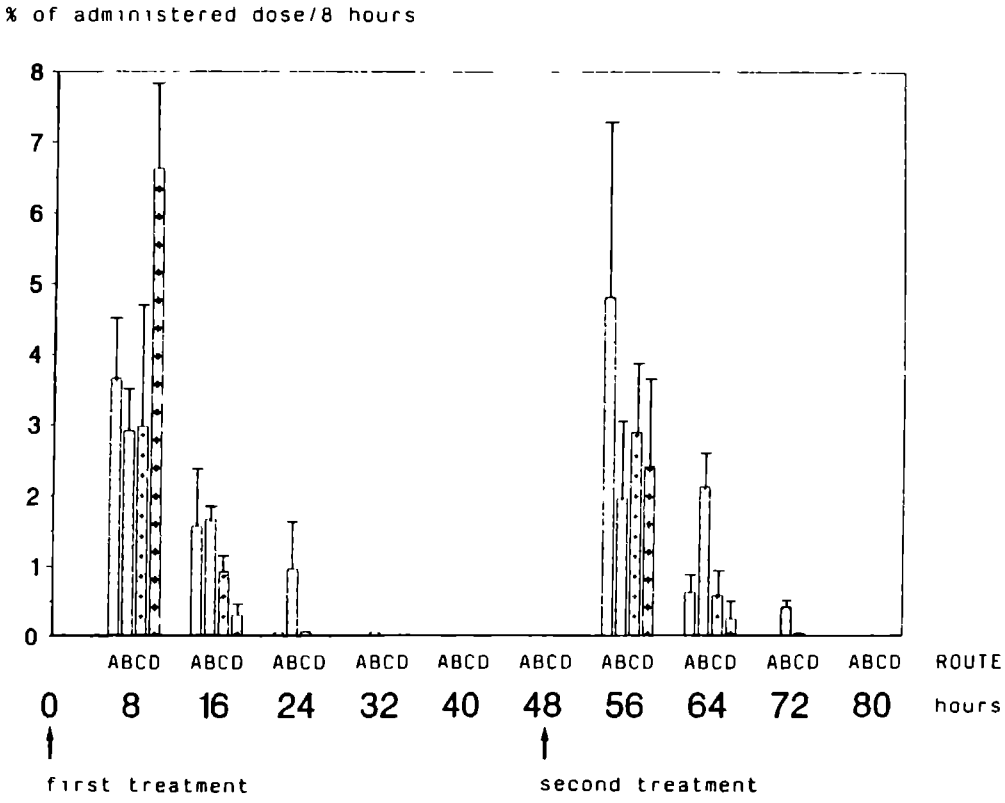

Table 1. Cumulative excretion of CP in urine of rats in the first 32 h after intratracheal, dermal, oral and intravenous treatment with CP[a].

route of administration	first treatment		second treatment	
	excretion (μg)	% dose	excretion (μg)	% dose
intratracheal	11.9 ± 2.5	5.2 ± 1.0	12.5 ± 3.4	5.5 ± 1.6
dermal	13.6 ± 0.3	5.6 ± 0.4	11.2 ± 4.7	4.5 ± 1.6
oral	8.4 ± 3.7	4.0 ± 2.0	7.6 ± 2.4	3.6 ± 1.2
intravenous	14.9 ± 2.8[b]	6.9 ± 1.1[b]	6.1 ± 3.3	2.7 ± 1.5

[a] mean of groups of three rats ± S.D.
[b] only two rats, because of loss of one urine sample

Discussion

Some investigators have found that the cumulative excretion of CP in urine of CP-treated rats ranged from 11 to 35% of the dose after intraperitoneal administration of therapeutic amounts. [15,16] The cumulative CP excretion appeared to increase with dose but the rate of excretion seemed to be independent of dose.After treatment with 1 mg kg^{-1} body weight, the cumulative excretion was below 7% (Table 1). The lowest cumulative excretion was observed after oral administration, probably because of a first pass effect. Such an effect has been found in patients when oral treatment was compared with intravenous injection.[17]

Cumulative excretion showed some difference between the first and a second intravenous treatment. The first treatment resulted either in an inhibition of excretion or an induction of biotransformation. Some human studies suggested induction by repeated treatments with CP,[18,19] while in other studies no such effect was observed.[20,21]

Our results, along with those of Hirst et al.[11] in dermal-treated volunteers, demonstrate that CP easily penetrates the skin. Therefore, dermal exposure must be considered as a possible exposure route for CP.

Hirst et al. also found a higher rate of excretion of CP in urine of nurses after handling CP in comparison with dermal-treated volunteers.[11] Their results suggested that the route of exposure of nurses may differ from that of the volunteers. The velocity with which CP appeared in urine implied that it was absorbed soon after its' preparation and it was likely that CP entered the

circulation following inhalation. Our results show that after intratracheal administration, CP must be absorbed easily because it appeared in urine rather quickly. Although the experimental conditions were different, our results are in agreement with those of Hirst et al.[11]

In conclusion, the results show little difference in excretion rate and cumulative urinary excretion of CP in rats after the first or after the second intratracheal, dermal, oral or intravenous treatment. Excretion was rapid and almost complete within 8-16 h after treatment, except that excretion was delayed after dermal application. These results suggest that intake of CP by inhalation and absorption via the skin, which are expected to be the most important routes of occupational exposure to cytostatic drugs, are possible and can be determined by the measurement of CP in urine.

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4.2

Determination of cyclophosphamide metabolites by gas chromatography and thermionic specific detection Interindividual differences in hepatic biotransformation of cyclophosphamide in man *in vitro*

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Abstract

Sensitive methods for the determination of the cyclophosphamide metabolites nornitrogen mustard, 4-ketocyclophosphamide and carboxyphosphamide are presented. After liquid-liquid extraction and derivatization, the metabolites are determined by gas chromatography and thermionic specific detection. The methods were used to study the in vitro biotransformation of cyclophosphamide with S-9 liver fractions of human donors. The results show large interindividual differences in the formation of nornitrogen mustard and carboxyphosphamide. 4-Ketocyclophosphamide was not detected.

Introduction

Cyclophosphamide (CP) is an alkylating antineoplastic agent with activity against a variety of human tumours [1]. CP is also used in the treatment of autoimmune diseases and during renal and bone marrow transplantations [1]. The activity of CP is caused by its metabolites [1]. During bioactivation by hepatic cytochrome P-450, 4-hydroxycyclophosphamide is formed which is in equilibrium with its ring-opened tautomer aldophosphamide (Fig.1). Both metabolites are detoxified by isoenzymes of the NAD-linked aldehyde dehydrogenase and aldehyde oxidase resulting in the formation of carboxyphosphamide (CAR) and 4-ketocyclophosphamide (KCP), respectively.

ß-Elimination of acrolein from aldophosphamide yields phosphoramide mustard, a directly active alkylating species which reacts with DNA Cleavage of phosphoramide mustard results in the formation of nornitrogen mustard (NNM) Acrolein binds covalently to proteins. Most CP metabolites are ultimately eliminated by renal excretion

In recently published studies, we have determined non-metabolised CP in urine samples of occupationally exposed hospital workers handling antineoplastic agents [2-5] For the determination of low concentrations in urine a sensitive gaschromatographic-mass spectrometric method was developed [6] In urine samples of many hospital workers CP was found However, little is known about interindividual differences in biotransformation of this agent Therefore, the in vitro biotransformation of CP was studied using (non)induced S-9 liver fractions. The rate of formation of the toxic metabolite NNM and the non-toxic metabolites KCP and CAR were measured We were especially interested in the balance between toxification (NNM formation) and detoxification (KCP + CAR formation) which is expressed by the toxic/non-toxic metabolites ratio NNM/(KCP + CAR)

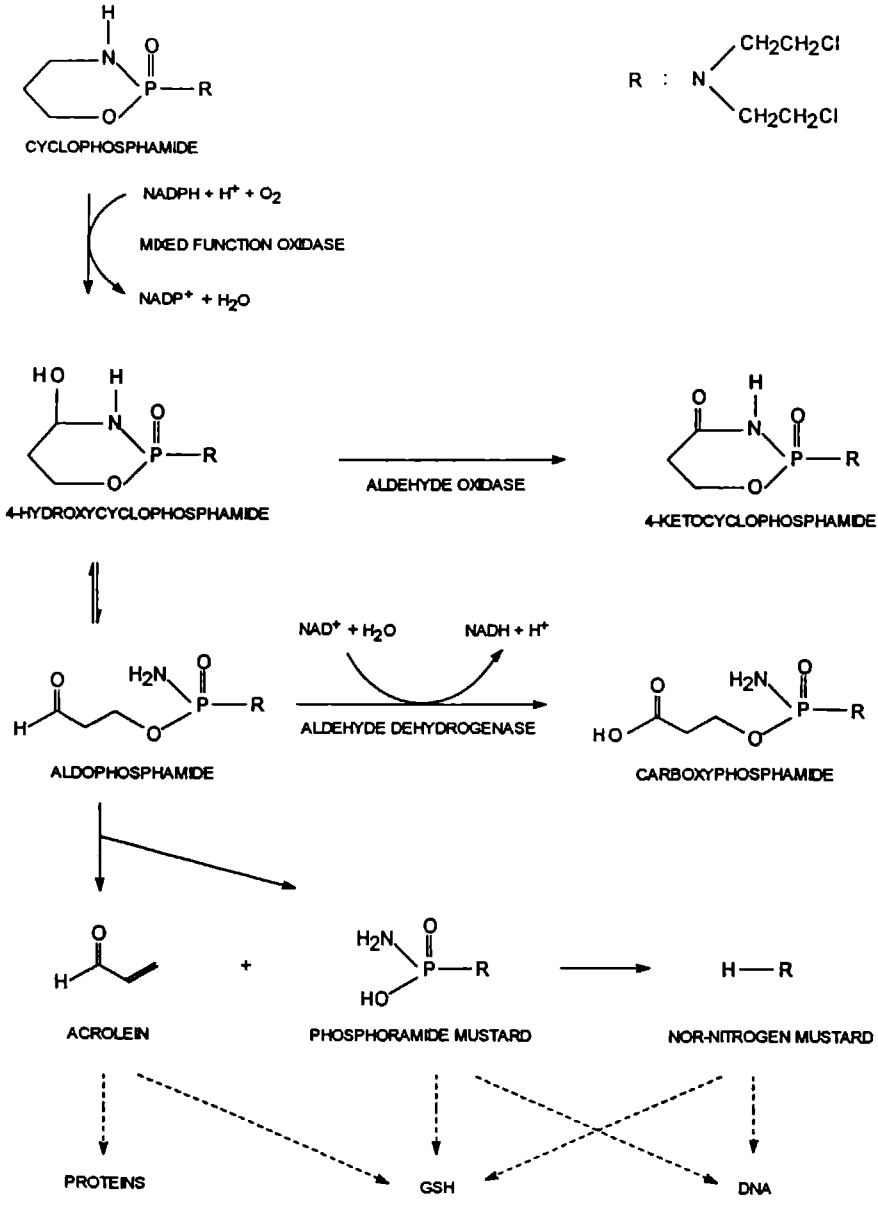
The number of chromatographic methods available for the monitoring of CP metabolites is limited [7] Therefore, new methods for the determination of KCP, CAR and NNM in incubation mixtures are developed. After liquid-liquid extraction and derivatization, the metabolites are determined with gas chromatography and thermionic specific detection NNM was determined after derivatization with benzenesulphonyl chloride KCP and CAR were determined after derivatization with N,N-dimethylformamide dimethyl acetal

Experimental

Chemicals

CP, 4-hydroxyperoxycyclophosphamide, trophosphamide (TRO), CAR and KCP were gifts from ASTA-Medica (Frankfurt am Main, Germany). Glucose-6-phosphate, sucrose and aniline were purchased from Sigma (St. Louis, MO, USA). 7-Ethoxy- and 7-pentoxyresorufin were from Boehringer (Mannheim, Germany). Nornitrogen mustard HCl (NNM) and resorufin were from Aldrich (Milwaukee, WI, USA) and benzenesulphonyl chloride, EDTA and 4-morpholinepropanesulphonic acid (MOPS), were obtained from Janssen Chimica (Beerse, Belgium) N,N-dimethylformamide dimethyl acetal was purchased from Chrompack (Middelburg, Netherlands) NADP was from USB (Cleveland, OH, USA) All other chemicals were of the highest purity obtainable.

Fig. 1. Schematic presentation of the biotransformation of cyclophosphamide showing the most important reactions and metabolites (after Sladek [1]).



Human livers

Pieces of human donor livers were used. They were kindly obtained from Dr. W.H.M. Peters, Division of Gastrointestinal and Liver Diseases, University of Nijmegen, Netherlands. Donor livers were made available anonymously. Consequently, limited information on the age and sex and no information about smoking and drinking habits was available. Their characteristics are presented in Table 1.

Preparation of the S-9 liver fractions

Liver homogenates were prepared (20%, w/v) in SETH-buffer (0.25 *M* sucrose, 2 *mM* EDTA and 10 *mM* Tris-HCl, pH = 7.4) with a Teflon-glass homogenizer. After centrifugation at 9000 g for 20 min, the floating fat layer was removed. The S-9 liver fraction was divided into a number of samples, frozen in liquid nitrogen and stored at -80°C until use.

In vitro incubations

The frozen S-9 liver fractions were thawed quickly at 37°C immediately prior to use and kept on ice. The incubation mixture contained 44 *mM* MOPS buffer pH = 7.4, 2 *mM* NADP, 5 *mM* $MgCl_2.6H_2O$, 2 *mM* glucose-6-phosphate, 1 *mM* EDTA, and an amount of S-9 liver fraction based on 3.5 mg protein. After a preincubation of 5 min at 37°C, the incubation was started by adding 1.5 μmol CP. The final incubation volume was 1 ml. The mixture was mildly shaken during the (pre)incubation at 150 rpm. The incubation was stopped after 30 min by the addition of 75 μl of a 6 M HCl solution. The reaction mixture was immediately cooled on ice-water. All incubations were carried out double in triplicate. One series in triplicate was used for the determination of CAR and KCP. The other was used for the determination of NNM.

Enzyme measurements

Total cytochrome P-450 was determined from the dithionite-difference spectrum according to Rutten et al. [8]. Protein concentrations were measured with the method of Bradford using crystalline bovine serum albumin as standard [9]. 7-Ethoxyresorufin-O-deethylase (EROD) activity was determined according to Burke et al. [10] and 4-aniline-hydroxylase (AH) was measured according to Ishidate et al. [11]. Enzyme and total cytochrome P-450 measurements were performed in duplicate and protein concentrations were measured in triplicate.

Sample preparation and gas chromatographic analysis of the cyclophosphamide metabolites

CAR and KCP

After the incubation was stopped, 45 µL 10 *M* NaOH and 100 µl 1 *M* MOPS buffer pH = 8.0 were added. Excess of CP was removed by 4 extractions with 7 ml of diethyl ether by shaking for 5 min to avoid interference during the analysis. The ether layers were discarded and 200 µl 6 M HCl was added. The mixture was extracted twice with 4.5 ml ethyl acetate by shaking during 5 min. The ethyl acetate layers were combined in conical tubes with screw caps and dried under nitrogen at 40°C. After addition of 700 µl toluene, 25 µl N,N-dimethylformamide dimethyl acetal and subsequently mixing, the tubes were closed for derivatization for 35 min at 115°C. The samples were cooled to room temperature and dried again under nitrogen at 50°C. Thereafter 200 µl toluene, containing 1 µg TRO (internal standard for controlling the sensitivity of the detector) was added, followed by sonification for 5 min. A 1-µl sample was injected.

Gas chromatography with thermionic specific detection was performed on a Varian 3400 GC that was controlled by a Varian GC Star Workstation on a Grid 386 personal computer. Samples were injected splitless. A 15-m DB-5 column (J&W, Folsom, CA, USA) was used with 0.32 mm internal diameter and 0.25 µm film thickness. The injector temperature was 320°C. The initial oven temperature was 85°C. After 1 min, the temperature was increased to 280°C following 4 segments (85-170°C, 20°C/min; 170-190°C, 5°C/min; 190-210°C, 10°C/min; 210-280°C, 20°C/min) where it remained constant for 6 min. Helium was used as carrier gas [column inlet pressure 12 p.s.i. (ca. $8.2 \cdot 10^2$ Pa)]. The thermionic specific detector was set at 300°C. For quantification the peak area ratios CAR/TRO and KCP/TRO were calculated. Quantification of the CAR and KCP derivatives was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing CAR (0, 5, 10, 20, and 25 nmol) and KCP (0, 13, 26, 38, and 51 nmol) dissolved in a blank incubation mixture. The reference samples containing CAR and KCP underwent the whole sample preparation procedure.

NNM

The incubation was stopped after 15 s, and 45 µl 10 M NaOH and 100 µl 1 M MOPS buffer pH = 8.0 were added. After 22 h at 37°C, 4.5 ml diethyl ether and 5 µl benzenesulphonylchloride were added successively and the mixture was shaken during 90 min, and centrifuged during 5 min at 2500 rpm (1500 g). The ether layers were removed and dried under nitrogen at 30°C. After addition of 1 ml water, the solution was extracted twice with 2 ml *n*-hexane. The hexane layers were removed and dried under nitrogen at 40°C. Finally, the extract was dissolved in 200 µl toluene containing 1 µg TRO (internal

standard for controlling the sensitivity of the detector). A 1 μl sample was injected.
For the analysis of NNM the same equipment and conditions were used as for the analysis of CAR and KCP, except for the oven programme. The initial oven temperature was 85°C. After 1 min, the temperature was increased by 15°C/min to 280°C, where it remained constant for 6 min. For quantification the peak area ratio NNM/TRO was calculated. Quantification of the derivatized NNM and TRO was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing 4-hydroxyperoxycyclophosphamide (0, 25, 50, 100, and 200 nmol) dissolved in blank incubation mixtures. For the determination of NNM we were not able to use calibration curves constructed from the analysis of freshly prepared reference samples containing NNM itself. It appearred that NNM was not formed within the incubation period but in a later stage out of metabolites generated during the incubation (Fig. 1). Therefore, the incubation mixture was put at 37°C during 22 h and 4-hydroxyperoxycyclophosphamide, which was completely converted to NNM, was preferred for the construction of the calibration curve instead of NNM itself.

Statistical analysis
Statistical analysis was carried out on a personal computer using InStat 1.1 as software package. Correlations were quantified with the Pearson correlation coefficient *r*. *P*-values (two-tailed) below 0.05 were considered to be of statistical significance.

Results and discussion

We have developed methods for the determination of the CP metabolites KCP, CAR and NNM in incubation mixtures. Prior to analysis, KCP and CAR were extracted from the incubation mixture with ethyl acetate and methylated with N,N-dimethylformamide dimethyl acetal. Fig. 2 shows typical chromatograms of derivatized extracts of a blank incubation mixture spiked with KCP, CAR and TRO (internal standard) (A); a blank incubation mixture spiked with TRO (B); and an incubation mixture containing a S-9 fraction of a human donor liver (C). The retention times of KCP, CP, CAR, and TRO were 6.8, 6.9, 7.2 and 8.9 min, respectively. The C.V. of the retention times was below 0.7%. In order to quantify the amount of KCP exactly, it was necessary to remove excess of CP. This was done by four extractions with diethyl ether prior to the extraction of the acidified incubation mixture with ethyl acetate. Finally, KCP and CP were completely separated and no interference with other compounds was observed. NNM was derivatized with benzenesulphonylchloride in diethyl ether according to the so-called Hinsberg-test resulting in the formation of a

sulfonamide [12]. Fig. 3 shows typical chromatograms of derivatized extracts of a blank incubation mixture spiked with NNM (in situ formed out of 4-hydroxyperoxycyclophosphamide) and TRO (internal standard) (A); a blank incubation mixture spiked with TRO (B); and an incubation mixture containing a S-9 fraction of a human donor liver (C). The retention times of NNM, CP, and TRO were 7.6, 7.8 and 9.0 min, respectively. The C.V. of the retention times was below 0.2%). No interference of NNM, CP and TRO with other compounds was observed. All calibration curves were linear, with a coefficient of correlation of a least 0.99. The limits of detection for CAR, KCP and NNM were 0.5, 0.5 and 0.1 nmol/ml incubation mixture (2.5, 2.5 and 0.5 pmol on column). The intra-assay precision (mean relative standard deviation of all triplicate incubations) was about 9% for NNM and 16% for CAR. The recovery of the extraction procedures is almost 100%.

Fig. 2. Chromatograms of ethyl acetate extracts of incubation mixtures after derivatization with N,N-dimethylformamide dimethyl acetal. Peaks (retention time): KCP = 4-ketocyclophosphamide (6.8 min); CP = cyclophosphamide (6.9 min); CAR = carboxyphosphamide (7.2 min); TRO = trophosphamide (internal standard) (8.9 min). (A) Blank incubation mixture spiked with KCP (26 nmol/ml incubation mixture), CAR (10 nmol/ml incubation mixture) and TRO (15 nmol/ml toluene). (B) Blank incubation mixture spiked with TRO (15 nmol/ml toluene). (C) Incubation mixture containing S-9 liver fractions of liver 4.

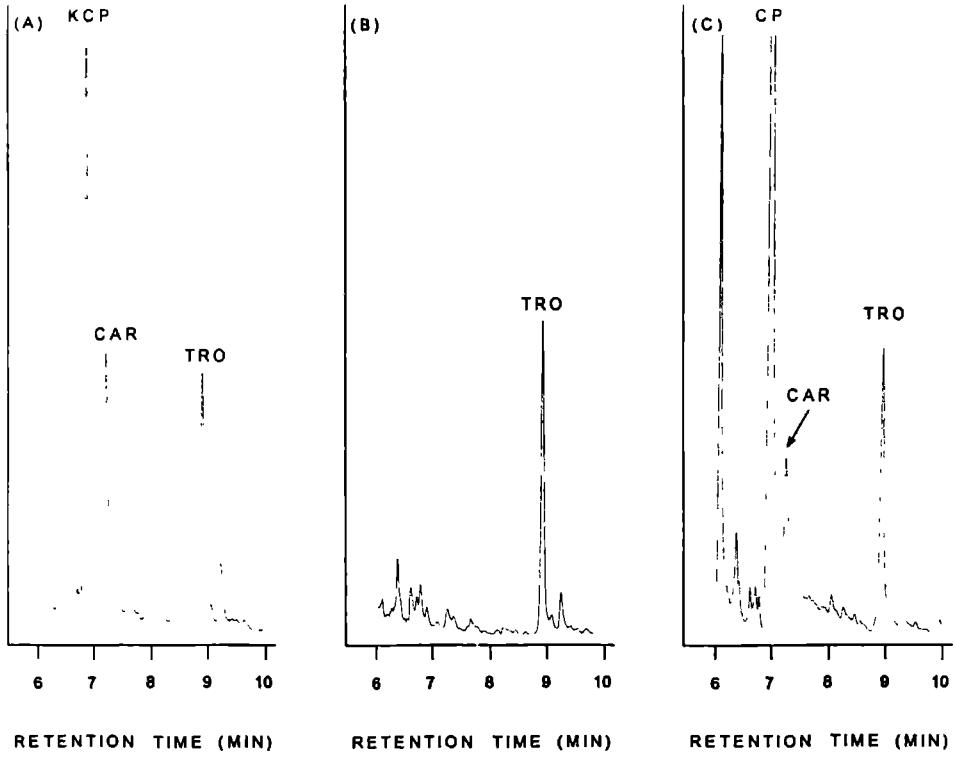
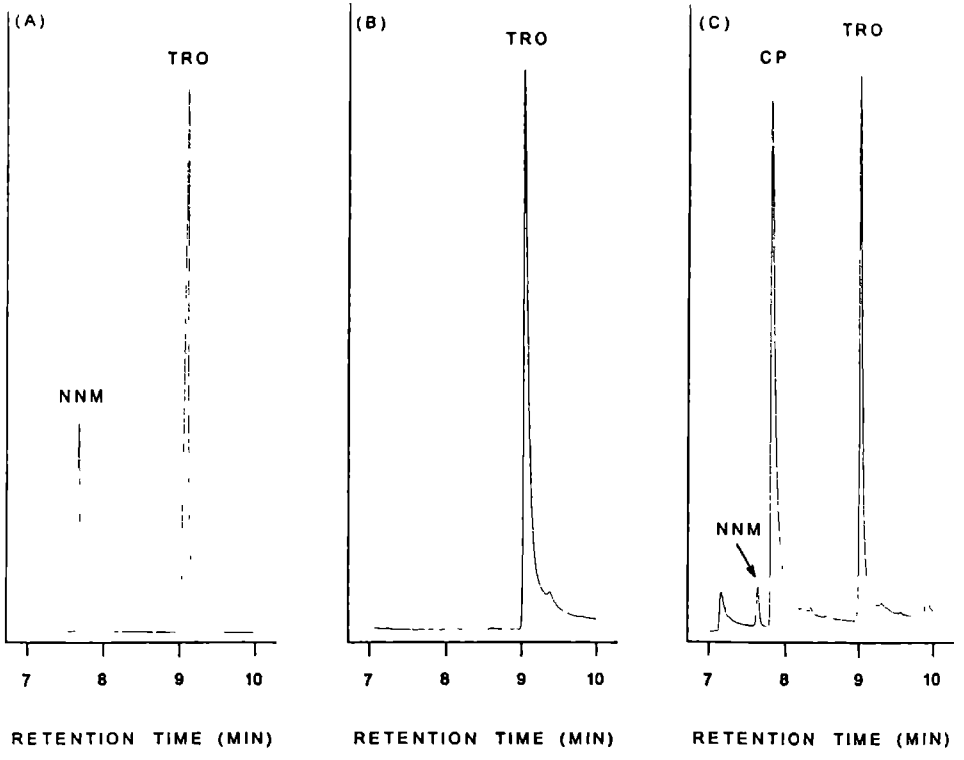


Fig. 3. Chromatograms of diethyl ether extracts of incubation mixtures after derivatization with benzenesulphonylchloride. Peaks (retention time): NNM = nornitrogen mustard (7.6 min); CP = cyclophosphamide (7.8 min); TRO = trophosphamide (internal standard) (9.0 min). (A) Blank incubation mixture spiked with NNM (100 nmol/ml incubation mixture) and TRO (15 nmol/ml toluene). (B) Blank incubation mixture spiked with TRO (15 nmol/ml toluene). (C) Incubation mixture containing S-9 liver fractions of liver 7.



Large differences in total P-450 content and EROD and AH activities were observed among the liver fractions of the donors (Table 1). Total P-450 content ranged from 0.08 to 0.22 nmol/mg protein. EROD and AH activity ranged from 2.7 to 12.9 and from 0.06 to 0.32 pmol/min per mg protein, respectively. Large differences were also found in NNM, CAR and total metabolite formation and NNM/CAR ratio after incubation of CP with human S-9 liver fractions (Table 2). In none of the incubation mixtures KCP was detected. The highest amount of NNM was formed by liver 1. The NNM

formation rate was eight times higher when compared to liver 7 having the lowest NNM formation rate. Liver 4 showed the highest CAR formation rate. This was fifteen times higher when compared to liver 6, having the lowest rate. Liver 4 also showed the highest NNM + CAR formation rate. This was eight times higher when compared to liver 6 with the lowest NNM + CAR formation. Liver 1 showed the highest NNM/CAR ratio. This was four times higher when compared to liver 4, the lowest one. It should be mentioned that the livers 1 and 4 with the highest NNM + CAR formation differed substantially in NNM/CAR ratio. Comparable results were found for the livers 6 and 7 with the lowest NNM + CAR formation.

Table 1. Total P-450 content, EROD and AH activities in human S-9 liver fractions.

liver	sex	age	total P-450 (nmol mg^{-1} protein)	EROD[a] (pmol min^{-1} mg^{-1} protein)	AH[b] (pmol min^{-1}mg^{-1} protein)
1	?[c]	?	0.09	4.7	0.17
2	male	16	0.11	3.6	0.18
3	?	?	0.09	3.7	0.13
4	?	20	0.22	4.0	0.32
5	male	47	0.09	8.0	0.15
6	?	?	0.12	12.9	0.19
7	?	?	0.08	2.7	0.06
Mean ± S.D.			0.11 ± 0.05	5.6 ± 3.6	0.17 ± 0.08

[a] 7-ethoxyresorufin-O-deethylase
[b] 4-aniline-hydroxylase
[c] sex and age are not known

The results show large differences in the biotransformation of CP. Comparable results were found by Chang et al. although microsomes of human donor livers were used instead of S-9 liver fractions as was done in our study [13]. Besides, they studied CP 4-hydroxylase activity fluorometrically while we have determined CP metabolites with gas chromatography. They found that P-4501A1, 1A2 and 2E1 did not contribute to the hydroxylation of CP. In our study, we did not find a correlation between EROD (P-4501A1) activity and NNM, CAR, total metabolite formation and the NNM/CAR ratio. On the con-

trary, we found a correlation between CAR and total metabolite formation and AH (P-4502E1) activity ($r=0.8373$, $P=0.0187$ and $r=0.7954$, $P=0.0325$, respectively). NNM formation and the NNM/CAR ratio were not correlated. Unfortunately, it is not possible to establish the causal factor of the large differences in EROD and AH activity between the individual human S-9 liver preparations since no more data of the donors was available. Enhanced activity of EROD and AH do not per se reflect the induction of specific enzymes caused, for instance, by the life style factors smoking or alcohol. Genetic factors may also be of importance with respect to P-450 isoenzyme activities involved in the biotransformation of CP.

Table 2. Formation of NNM and CAR after incubation of cyclophosphamide with human S-9 liver fractions[a].

liver	NNM	CAR	NNM + CAR	NNM/CAR ratio
1	89	39	128	2.3
2	40	21	61	1.9
3	18	14	32	1.3
4	69	117	186	0.6
5	22	23 (n=2)	45	1.0
6	15	8 (n=2)	23	1.9
7	11	14	25	0.8
Mean ± S.D.	38±31	34±38	71±61	1.4±0.7

[a] values of NNM and CAR formation are means ± S.D. of duplicate (n=2) or triplicate incubations (pmol/min per mg protein)

The large interindividual differences in especially the formation of NNM and CAR and the NNM/CAR ratio between the liver S-9 samples suggest large interindividual differences in the toxicity of CP due to differences in biotransformation. A high NNM/CAR ratio may be predictive for a relatively high level of DNA-alkylating metabolites in vivo. Otherwise a low NNM/CAR ratio may be predictive for the formation of detoxified metabolites.
We may assume that comparable differences in biotransformation will occur in man in vivo, for instance in treated patients and in occupationally exposed workers. This means that it is not possible to estimate the exact individual dose of CP in occupationally exposed workers on basis of their individual

urinary excretion of CP. Therefore, we have applied the developed methods for the determination of KCP, CAR and NNM in urine of hospital workers. Unfortunately, the methods were not appropriate because we were not able to extract the metabolites and to derivatize them. Hence, only the method for the determination of CP in urine of hospital workers can be used to establish occupational exposure to CP and to estimate a group based exposure level.

Acknowledgement

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4.3

Influence of Aroclor 1254, phenobarbital, ß-naphthoflavone, and ethanol pretreatment on the biotransformation of cyclophosphamide in male and female rats

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Abstract

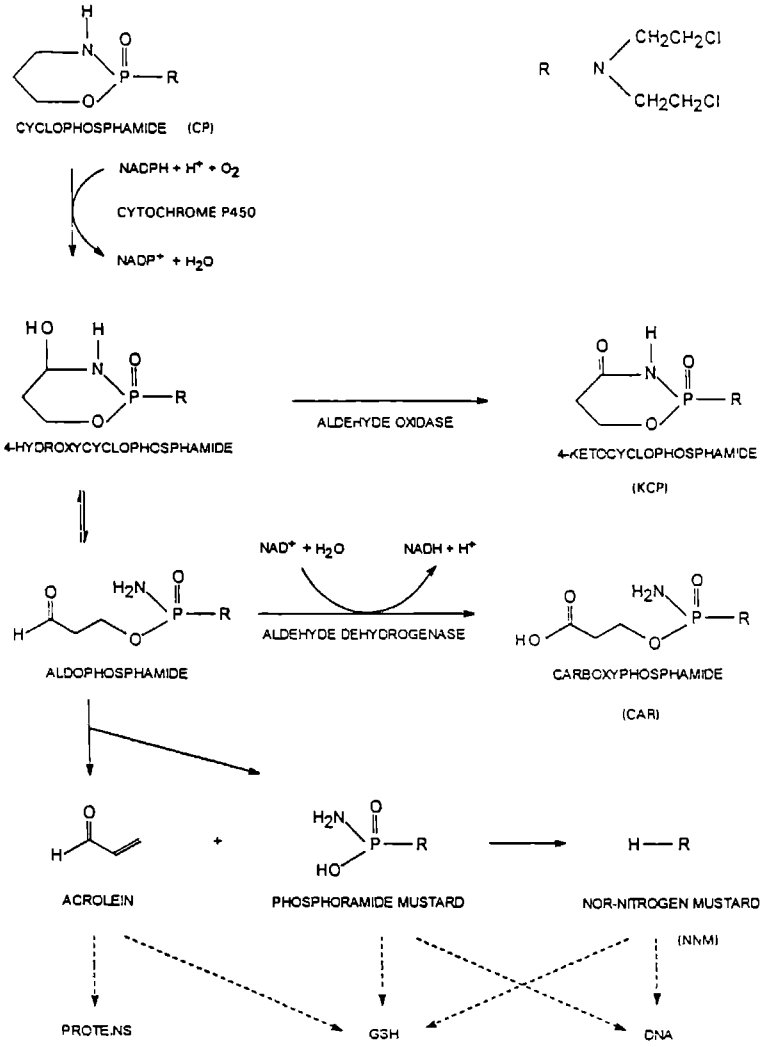
The aim of the present study is to investigate the influence of the environmental factors, smoking and alcohol, on the biotransformation of cyclophosphamide (CP) in the rat in vivo and in vitro with S9 liver fractions. The biotransformation of CP was studied by the determination of the CP-metabolites, nor-nitrogen mustard (NNM), 4-ketocyclophosphamide (KCP), and carboxyphosphamide (CAR). The effect of the environmental factors, smoking and alcohol consumption, on the biotransformation enzymes was mimicked by pretreatment of rats with ß-naphthoflavone and ethanol, respectively. Rats treated with olive oil and water served as controls and rats pretreated with Aroclor 1254 and phenobarbital were used as positive controls. The influence of sex and supplementation with NAD and GSH, mimicking a biological variation in NAD and GSH levels in rat and human liver, was also studied. Pretreatment of rats with Aroclor 1254 decreased the excretion of unmetabolized CP in urine, most likely due to an enhanced biotransformation. The in vitro hepatic biotransformation of CP in rats was strongly influenced by sex, by supplementation with NAD and GSH, and by pretreatment with the enzyme-inducers, phenobarbital and Aroclor 1254. No influence of pretreatment with the enzyme-inducers, ß-naphthoflavone and ethanol, was found. The results suggest that the influence of the environmental factors, alcohol consumption and smoking, on the biotransformation of CP in man will be negligible.

Introduction

Cyclophosphamide (CP) is an important antineoplastic drug used in the chemotherapy of cancer and autoimmune diseases. The activity of CP is caused by its metabolites (Sladek, 1988). During bioactivation by hepatic cytochrome P450, 4-hydroxycyclophosphamide is formed which is in equilibrium with its ring-opened tautomer aldophosphamide (Fig. 1). Both metabolites are detoxified by isoenzymes of the NAD-linked aldehyde oxidase and aldehyde dehydrogenase resulting in the formation of 4-ketocyclophosphamide (KCP) and carboxyphosphamide (CAR), respectively. Toxification occurs after ß-elimination of acrolein from aldophosphamide to yield phosphoramide mustard which is further converted to nor-nitrogen mustard (NNM). Phosphoramide mustard is responsible for the DNA-alkylating activity resulting in the cytotoxic effect of CP. Acrolein binds covalently to proteins. Most CP(metabolites) are ultimately eliminated by renal excretion.
In several recently published studies we have investigated the exposure of hospital workers to CP (Sessink et al., 1992a,b; 1994a,b; 1995b). It was found that during drug handling the workers were exposed to this compound. Despite the introduced guidelines and protective measures, we were able to detect CP in their urine. It was concluded that uptake of CP due to occupational exposure did happen. The question is to what extent the amounts of CP excreted in urine are a measure for the uptake of the drug. More precisely, what is the relationship between uptake and urinary excretion of CP. Several processes such as the biotransformation, resulting in transformation of the parent compound in metabolites, are involved. Differences in biotransformation are genetically determined but are also influenced by chemical exposure from the environment among which so-called life style factors, that may cause induction or inhibition of enzymes involved in the biotransformation of toxic compounds such as CP (Fournier and Thomas, 1986).
In the present study the in vitro biotransformation of CP was investigated using S9 liver fractions of pretreated and control rats. The biotransformation of CP was studied by the determination of the CP-metabolites, nor-nitrogen mustard (NNM), 4-ketyocyclophosphamide (KCP), and carboxyphosphamide (CAR). The effects of the environmental factors, smoking and alcohol, on biotransformation enzymes were mimicked by pretreatment of rats with ß-naphthoflavone and ethanol, respectively (Pelkonen et al., 1986; Sesardic et al, 1987). The results were compared with the results obtained from control rats (olive oil and water pretreatment, respectively) and from rats pretreated with Aroclor 1254 and phenobarbital, respectively (positive controls). The influence of sex and supplementation with NAD and GSH, mimicking a biological variation in NAD and GSH levels in rat and human liver, was also studied. We were especially interested in the balance between toxication and

detoxication which is expressed by the toxic/non-toxic metabolites ratio NNM/(KCP + CAR).

Fig. 1. Abridged scheme of the biotransformation of CP showing only the currently recognized biologically important reactions and metabolites (after Sladek, 1988).



Several investigators have studied the in vitro hepatic biotransformation of CP in rats after pretreatment with phenobarbital, Aroclor 1254, ß-naphtoflavone and 3-methylcholanthrene (Sladek, 1988; Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). In our study, we were able to detect specific CP metabolites using recently developed gas chromatographic methods after the incubation of CP with S9 liver fractions instead of microsomes or purified cytochrome P450 (iso)enzymes (Sessink et al., 1995a).
The biotransformation of CP was also studied in vivo by determination of unmetabolized CP in urine of rats pretreated with Aroclor 1254. CP was administered by intravenous injection. In addition, dermal application was chosen to mimic the possible dermal uptake as expected during occupational activities of hospital workers (Hirst et al., 1984; Sessink et al., 1994a).

Methods

Animal pretreatment for the determination of CP in urine
Four Groups of three male random-bred Wistar rats (Cpb:WU (SPF), body wt about 215 g) were housed individually in stainless-steel metabolism cages and had free access to tap water and RMH food pellets (Hope Farms BV, Woerden, The Netherlands). Urine samples and faeces were collected separately. Two groups of rats were pretreated with Aroclor 1254 (Alltech, Deerfield, IL, USA) in olive oil (i.p., 500 mg/kg body wt, 5 days before CP administration) or olive oil as control (i.p., 2 ml/kg body wt). CP (ASTA-Medica, Frankfurt am Main, Germany) was administered by dermal application or intravenous injection (penis vene) to a group of Aroclor 1254-pretreated rats and to a group of control rats (1 mg/kg body wt). For dermal application, a glycerol suspension was applied on about 2 cm^2 of the close-shaven skin of the neck. Urine samples were collected on ice water for 24 h. A dose of 1 mg CP/kg body wt is about 10000 times higher when compared to the occupational exposure of hospital workers to CP (3.6-18 μg CP/day) (Sessink et al., 1995b).

Animal pretreatment for the in vitro incubations
Six Groups of three male and six groups of three female random bred Wistar rats (Cpb:WU (SPF), body wt about 190 g) were used. The animals were housed per group. They had free access to tap water and RMH food pellets (Hope Farms BV, Woerden, The Netherlands). Groups of male and female rats were pretreated with Aroclor 1254 in olive oil (i.p., 500 mg/kg body wt, 5 days before preparation of the S9 liver fraction), phenobarbital (OPG, Utrecht, The Netherlands) in saline (i.p., male rats: 75 mg/kg body wt, female rats: 50 mg/kg body wt, during 4 days before preparation of the S9 liver fraction), ß-naphthoflavone (Janssen, Beerse, Belgium) in olive oil (i.p., 40 mg/kg body wt, during 3 days before preparation of the S9 liver fraction) and olive oil as

control (i.p., 2 ml/kg body wt). Groups of ethanol pretreated male and female rats were obtained by giving tap water containing 15% ethanol for 6 weeks The corresponding control rats received tap water without ethanol (van de Wiel et al., 1990) The rats were anaesthetized with pentobarbital and finally killed by cervical dislocation.

Preparation of the S9 liver fractions

After cervical dislocation, the livers were perfused in situ with 0.9% NaCl (w/w), completely removed, weighed and immediately cooled in ice-cold SETH buffer (0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH 7 4) Liver homogenates were prepared (20%, w/v) in SETH buffer with a teflon-glass homogenizer After centrifugation at 9000 x *g* for 20 min, the floating fat layer was removed. The S9 liver fraction was divided in a number of samples, frozen in liquid N_2 and stored at -80°C until use

In vitro incubations

The frozen S9 liver fractions were thawed quickly at 37°C immediately prior to use and kept on ice. The incubation mixture contained 44 mM MOPS buffer pH 7.4, 2 mM NADP, 5 mM $MgCl_2 \cdot 6H_2O$, 2 mM glucose-6-phosphate, 1 mM EDTA, 4 mM NAD, 5 mM GSH, and an amount of S9 liver fraction based on 0 35 nmol P450 After a preincubation of 5 min at 37°C, the incubation was started by adding 1.5 μmol CP. The final incubation volume was 1 ml The mixture was mildly shaken during the (pre)incubation at 150 rev /min The incubation was stopped after 30 min by the addition of 75 μl of a 6 M HCl solution. The reaction mixture was immediately cooled on ice-water All incubations were carried out double in triplicate One series in triplicate was used for the determination of CAR and KCP. The other was used for the determination of NNM (Sessink et al , 1995a).

Enzyme measurements

Total cytochrome P450 was determined from the dithionite-difference spectrum according to Rutten et al. (Rutten et al , 1987) Protein concentrations were measured by the Bradford method using crystalline bovine serum albumine as standard (Bradford, 1976) 7-Ethoxyresorufin-*O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-deethylase (PROD) activities were determined according to Burke et al. and aniline-hydroxylase (AH) activity was measured according to Ishidate et al (Burke et al., 1985, Ishidate et al., 1978). Enzyme and total cytochrome P450 measurements were performed in duplicate. Protein concentrations were measured in triplicate

Sample preparation and gas chromatographic analysis of CP in urine
After liquid-liquid extraction and derivatization with trifluoroacetic anhydride, CP was determined with gas chromatography/mass spectrometry (Sessink et al., 1991, 1992b).

Sample preparation and gas chromatographic analysis of the CP metabolites in the incubation mixture
After liquid-liquid extraction and derivatization, the metabolites are determined with gas chromatography and thermionic specific detection. NNM was determined after derivatization with benzenesulphonyl chloride. KCP and CAR were determined after derivatization with *N,N*-dimethylformamide dimethyl acetal (Sessink et al., 1995a).

Statistical analysis
Two-way analysis of variance (SAS procedure GLM) was used to study influence of pretreatment and administration route for the in vivo experiments. These data were analyzed using Statistical Analysis System version 6.08 (SAS Institute Inc., Cary, NC, USA). Unpaired two-tailed *T*-tests were used to study influence of pretreatment, sex, and supplementation with NAD and GSH for the in vitro incubations (Tables 2 and 3). Log-transformed data were used. InStat 1.1 was used as the software package. *P*-values below 0.05 were considered to be significant

Results

Effect of Aroclor 1254 pretreatment and administration route on the urinary excretion of unmetabolized CP
CP was excreted in urine of control rats within 16-20 h after intravenous and dermal administration. After pretreatment with Aroclor 1254, CP excretion was completed at 4-8 h after administration. The cumulative CP excretion as percentage of the dose administered (± S.D.) was lower in the Aroclor 1254-pretreated rats (intravenous administration: 1 4 ± 0.4, dermal application: 0 6 ± 0.1) compared to the olive oil-pretreated control rats (intravenous administration. 7 6 ± 1.2, dermal application: 5.8 ± 1.1) ($P<0.0001$). The cumulative CP excretion was also lower after dermal application (Aroclor 1254-pretreated rats: 0.6 ± 0.1; olive oil control rats. 5.8 ± 1.1) compared to intravenous administration (Aroclor 1254-pretreated rats: 1.4 ± 0 4; olive oil control rats: 7.6 ± 1.2) ($P=0.026$). It is concluded that pretreatment with Aroclor 1254 decreased the excretion of unmetabolized CP in urine of rats most likely due to an enhanced biotransformation of CP in vivo. The influence of the administration route was of minor importance indicating almost complete dermal absorption.

Effect of pretreatment with cytochrome P450 enzyme-inducers on enzyme activities

As was expected, EROD activity was substantially increased in both male and female rats after Aroclor 1254 pretreatment and to a lesser extent after ß-naphthoflavone pretreatment in comparison with the olive oil control rats (Table 1). EROD activity was slightly increased in male rats after phenobarbital pretreatment. PROD activity was slightly increased in male and female rats after phenobarbital and Aroclor 1254 pretreatment and in female rats after ß-naphtoflavone pretreatment. AH activity was slightly enhanced in male and female rats after Aroclor 1254 and ethanol pretreatment and in male rats after phenobarbital pretreatment.

Effect of pretreatment with cytochrome P450 enzyme-inducers on the biotransformation of CP

The rates of formation of NNM and CAR after incubation of CP with S9 liver fractions pretreated and control male and female rats, and the influence of supplementation with NAD and GSH are shown in Table 2 (Aroclor 1254, ß-naphthoflavone, and olive oil control) and Table 3 (phenobarbital, ethanol, and water control). In none of the incubation mixtures KCP was detected (<0.5 nmol/ml incubation mixture). Consequently, the formulas for the calculation of the total metabolite formation rate and the toxic/non-toxic metabolites ratio are simplified in NNM + CAR and NNM/CAR, respectively.

Without supplementation with NAD and GSH

The results show that Aroclor 1254 and phenobarbital pretreatment increased the formation rate of NNM in male rats and the formation rate of CAR in male and female rats compared to their corresponding controls. The formation rate of NNM and CAR was not affected after ß-naphthoflavone and ethanol pretreatment. The formation rate of especially NNM and to a lesser extent CAR is higher in male than in female rats for all pretreatments and controls. Consequently, the total metabolite formation rate NNM + CAR and the NNM/CAR ratio is higher in male than in female rats.

Supplementation with NAD

Supplementation of the S9 incubation mixtures with NAD decreased the NNM formation rate in ß-naphtoflavone pretreated and in female olive oil-treated control rats. The NNM formation rate was not affected by the other pretreatments and in the water control group. On the contrary, the CAR formation rate was increased in male rats for all pretreatments and their corresponding control groups more or less compensating the decrease of the NNM formation rate. Consequently, the total metabolite formation rate NNM + CAR was not changed and the NNM/CAR ratio was decreased (except for female water control rats).

Table 1 *Effect of pretreatment on total cytochrome P450 content, EROD, PROD and AH activities in rat S9 liver fractions*[a]

pretreatment	total P450		EROD[b]		PROD		AH[d]	
	nmol/mg protein	fold[e] increase	pmol/min/ mg protein	fold[e] increase	pmol/min/ mg protein	fold[e] increase	pmol/min/ mg protein	fold[e] increase
male								
aroclor 1254	0 21	2 1	190	73	9 7	2 5	0 16	2 5
ß naphthoflavone	0 12	1 2	160	62	5 4	1 4	0 10	1 5
olive oil control	0 10	1[f]	2 6	1[f]	3 8	1[f]	0 06	1[f]
phenobarbital	0 24	2 4	29	5 3	15	4 5	0 12	2 5
ethanol	0 16	1 5	4 5	0 8	3 5	1 0	0 14	2 9
water control	0 10	1[f]	5 4	1[f]	3 3	1[f]	0 05	1[f]
female								
aroclor 1254	0 10	1 9	310	198	16	10	0 08	2 7
ß naphthoflavone	0 07	1 4	230	145	5 8	3 6	0 05	1 7
olive oil control	0 05	1[f]	1 6	1[f]	1 6	1[f]	0 03	1[f]
phenobarbital	0 06	0 6	2 8	0 9	8 1	6 5	0 03	0 6
ethanol	0 18	1 9	2 6	0 8	1 4	1 1	0 13	2 5
water control	0 10	1[f]	3 3	1[f]	1 3	1[f]	0 05	1[f]

[a] values are means of pooled S9 fractions of 3 rats
[b] EROD, 7 Ethoxyresorufin *O* deethylase
[c] PROD, 7 Pentoxyresorufin *O* deethylase
[d] AH 4 Aniline hydroxylase
[e] induction was compared to olive and water control
[f] for both male and female rats, olive oil and water were set at 1

Table 2 *Formation of NNM and CAR after incubation of CP with Aroclor 1254- and ß-naphthoflavone pretreated rat S9 liver fractions[a].*

	aroclor 1254		ß-naphthoflavone		olive oil control	
	♂	♀	♂	♀	♂	♀
without supplementation with NAD and GSH						
NNM	3300 ± 2000 [b* c***]	38 ± 23	400 ± 240 [c**]	17 ± 10	570 ± 350 [c***]	8 ± 5
CAR	270 ± 110 [b* c*]	57 ± 23 [b*]	53 ± 22 [c*]	14 ± 6	74 ± 30 [c*]	19 ± 8
NNM + CAR	3600	94	450	30	640	27
NNM/CAR RATIO	12 4	0 66	7 6	1 2	7 7	0 40
supplementation with NAD						
NNM	840 ± 510 [c***]	14 ± 9 [b***]	80 ± 48 [c**** d*]	0 [d***]	180 ± 120 [c****]	0 [d***]
CAR	1200 ± 480 [b* c*** d*]	78 ± 32	160 ± 64 [c*** d*]	10 ± 4 [b*]	330 ± 140 [c*** d*]	36 ± 15
NNM + CAR	2000	92	240	10	520	36
NNM/CAR RATIO	0 70	0 18	0 50	0	0 56	0
supplementation with NAD and GSH						
NNM	50 ± 31 [c**** e**]	0 [e***]	8 ± 5 [c*** e*]	0	14 ± 9 [c*** e*]	0
CAR	780 ± 320 [b* c**]	110 ± 44 [b*]	120 ± 48 [c*]	26 ± 11	190 ± 77 [c**]	30 ± 13
NNM + CAR	830	110	130	26	200	30
NNM/CAR RATIO	0 06	0	0 06	0	0 07	0

[a] values of NNM and CAR formation are means ± S D of 3 rats (pmol/min/mg protein)
[b] significantly different from olive oil control pretreated male or female rats
[c] significant difference between male and female rats concerning the indicated pretreatment and *(without) supplementation with NAD (and GSH)*
[d] significantly different from *without supplementation with NAD and GSH* concerning the indicated pretreatment and sex
[e] significantly different from *supplementation with NAD* concerning the indicated pretreatment and sex
* P<0 05, ** P<0 01, *** P<0 005, **** P<0 0005

Table 3 *Formation of NNM and CAR after incubation of CP with phenobarbital- and ethanol pretreated rat S9 liver fractions*[a]

	phenobarbital		ethanol		water control	
	♂	♀	♂	♀	♂	♀
without supplementation with NAD and GSH						
NNM	4700 ± 2800 [b* c***]	57 ± 34	610 ± 370 [c***]	14 ± 9	390 ± 240 [c**]	14 ± 9
CAR	390 ± 160 [b* c*]	94 ± 38 [b*]	60 ± 24	26 ± 11	94 ± 38 [c*]	18 ± 8
NNM + CAR	5100	150	670	40	490	32
NNM/CAR RATIO	11 9	0 61	10 2	0 54	4 2	0 74
supplementation with NAD						
NNM	2100 ± 1300 [b* c***]	30 ± 18	260 ± 160 [c**]	17 ± 10	220 ± 140	60 ± 36
CAR	2400 ± 960 [b*** c*** d**]	200 ± 82 [b***]	190 ± 76 [c* d*]	42 ± 17	300 ± 120 [c*** d*]	20 ± 9
NNM + CAR	4500	230	450	58	520	80
NNM/CAR RATIO	0 88	0 15	1 4	0 40	0 74	3 0
supplementation with NAD and GSH						
NNM	57 ± 35 [c** e***]	3 ± 2 [b* e*]	12 + 7 [c*** e**]	0 [e***]	10 ± 7 [c** e**]	0 [e****]
CAR	1700 ± 670 [b*** c***]	250 ± 98 [b***]	150 ± 60 [c*]	53 ± 22	210 + 86 [c**]	26 ± 12
NNM + CAR	1700	250	160	53	220	28
NNM/CAR RATIO	0 03	0 01	0 08	0	0 05	0

[a] values of NNM and CAR formation are means ± S D of 3 rats (pmol/min/mg protein)
[b] significantly different from water control pretreated male or female rats
[c] significant difference between male and female rats concerning the indicated pretreatment and *(without) supplementation with NAD (and GSH)*
[d] significantly different from *without supplementation with NAD and GSH* concerning the indicated pretreatment and sex
[e] significantly different from *supplementation with NAD* concerning the indicated pretreatment and sex
* P<0 05, ** P<0 01, *** P<0 005, **** P<0 0005

In addition, Aroclor 1254 pretreatment increased the formation rate of NNM in female rats and the formation rate of CAR in male rats compared to their corresponding controls. The formation rate of CAR was decreased after ß-naphtoflavone pretreatment in female rats. Phenobarbital pretreatment increased the formation rate of NNM in male rats and the formation rate of CAR in male and female rats compared to their corresponding controls. The formation rate of NNM and CAR was not affected after ethanol pretreatment. The formation rate of NNM and CAR is higher in male rats than in female rats for all pretreatments and the control groups (except the NNM formation rate in the water control group).

Supplementation with NAD and GSH

Supplementation of the S9 incubation mixtures with NAD and GSH decreased the NNM formation rate compared to NAD supplementation alone. The CAR formation rate was not affected. Consequently, the NNM/CAR ratio was decreased. The total metabolite formation rate NNM + CAR was only decreased in the male rats. This is due to the large reduction in the NNM formation rate in the male rats compared to the female rats. These results were not influenced by the pretreatment. Once again, the formation rate of NNM and CAR was higher in male rats than in female rats within each pretreatment.
In addition, Aroclor 1254 and phenobarbital pretreatment increased the formation rate of CAR compared to their corresponding controls. Phenobarbital pretreatment also increased the formation rate of NNM in female rats.

Discussion

The cumulative urinary CP excretion (24 h) was significantly decreased in Aroclor 1254-pretreated rats when compared to control rats. An enhanced in vivo biotransformation of CP is suggested and is confirmed by the results of the incubations in vitro which demonstrate an increase in total CP metabolite formation after pretreatment with Aroclor 1254. These results suggest the influence of external (chemical) factors on CP biotransformation in vivo. The cumulative CP excretion was lower after dermal application compared to intravenous administration. This suggests a higher biotransformation activity after dermal application than intravenous administration. Consequently more metabolites could be formed after dermal application than after intravenous administration. However, it is unknown whether this will result in more toxic or non-toxic metabolites.
Several investigators have studied the in vitro hepatic biotransformation of CP in rats after pretreatment with phenobarbital, Aroclor 1254, ß-naphtoflavone and 3-methylcholanthrene (Sladek, 1988; Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). The experiments were carried out with microsomes or

purified cytochrome P450 (iso)enzymes The biotransformation of CP was measured by application of several methods. However, no data about specific CP metabolite formation were presented It was shown that after phenobarbital pretreatment especially cytochrome P4502B1, contributed to the biotransformation of CP in the rat, whereas cytochromes P4502C6 and 2C11 were responsible for the biotransformation of CP in control rat liver (Clarke and Waxman, 1989, LeBlanc and Waxman, 1989) Recently, it was demonstrated that CP hydroxylation in man was preferentially catalyzed by liver microsomal cytochrome P4502B (Chang et al., 1993) It is suggested that induction of enzymes involved in CP biotransformation results in an increased therapeutic effect since more of the DNA-alkylating metabolite phosphoramide mustard is formed However, the absence of NAD-linked aldehyde dehydrogenase and aldehyde oxidase in incubations with microsomes and purified P450 systems might overestimate the formation of phosphoramide mustard because metabolites such as CAR and KCP could not be formed It was our aim to study the effect of enzyme induction and sex on the formation of toxic (NNM) and non-toxic (CAR and KCP) CP metabolites in rat liver S9 fractions.

In common with other investigators, we also found that the biotransformation of CP was increased after Aroclor 1254 and phenobarbital pretreatment (Sladek, 1988, Clarke and Waxman, 1989; LeBlanc and Waxman, 1989) However, the NNM/CAR ratio was slightly changed after Aroclor 1254 pretreatment but increased after phenobarbital pretreatment This is possibly caused by a higher increase in CAR formation due to induction of aldehyde dehydrogenase (Lindahl, 1992).

The effects of smoking and alcohol on biotransformation enzymes in the rat were mimicked by pretreatment of the rats with ß-naphthoflavone and ethanol, respectively These so-called life-style factors may cause induction or inhibition of enzymes involved in the biotransformation of toxic compounds such as CP (Fournier and Thomas, 1986) In the present study, no differences in the biotransformation of CP were observed after ß-naphthoflavone pretreatment (P4501A1 induction) and after ethanol pretreatment (P4502E1 induction) From these results it could be concluded that pretreatment of rats with ß-naphthoflavone and ethanol did not increase CP hydroxylation. Comparable results were found in in vitro experiments with microsomes of human donor livers which showed that P4501A1, 1A2 and 2E1 did not contribute to the hydroxylation of CP (Chang et al., 1993). In addition, we did not find a correlation between EROD activity and NNM, CAR, total metabolite formation and the NNM/CAR ratio in a human liver incubation study (Sessink et al , 1995a) On the contrary, we found a correlation between CAR and total metabolite formation and AH activity while NNM formation and the NNM/CAR ratio were not correlated

The formation rate of NNM and CAR will differ depending on the biological variation in NAD and GSH levels in rat and human liver This variation,

mimicked by supplementation with NAD necessary for the formation of CAR, increased the formation of this metabolite and decreased the formation of NNM as expected. In a corresponding way, NNM formation was decreased after supplementation with GSH. This is explained by conjugation of the electrophiles NNM and phosphoramide mustard.

We have found that the in vitro biotransformation of CP was strongly influenced by sex, and also by supplementation with NAD and GSH. It is concluded that pretreatment with the enzyme-inducers, ethanol and ß-naphtoflavone, did not influence the biotransformation of CP in the rat. Hence, it is tempting to conclude that alcohol consumption and smoking, mimicked by pretreatment of rats with ethanol and ß-naphthoflavone, respectively, will not affect CP biotransformation in man.

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Chapter 5

Risk assessment of occupational exposure to cyclophosphamide

5.1

Cancer risk assessment for health care workers occupationally exposed to cyclophosphamide

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Abstract

In the present study a cancer risk assessment of occupational exposure to cyclophosphamide (CP), a genotoxic carcinogenic antineoplastic agent, was carried out following two approaches based on (1) data from an animal study and (2) data on primary and secondary tumors in CP-treated patients. Data on the urinary excretion of CP in health care workers were used to estimate the uptake of CP, which ranged from 3.6 to 18 μg/day. Based on data of an animal study, cancer risks were calculated for a health care worker with a body weight of 70 kg and a working period of 40 years, 200 days a year (linear extrapolation). The lifetime risks (70 years) of urinary bladder cancer in men and leukemias in men and women were found to be nearly the same and ranged from 95 to 600 per million. Based on the patient studies, cancer risks were calculated by multiplication of the 10-year cumulative incidence per gram of CP in patients by the estimated mean total uptake in health care workers over 10 years, 200 days a year. The risk of leukemias in women over 10 years ranged from 17 to 100 per million using the secondary tumor data (linear extrapolation). Comparable results were obtained for the risk of urinary bladder tumors and leukemias in men and women when primary tumor data were used. Thus, on an annual basis, cancer risks obtained from both the animal and the patient study were nearly the same and ranged from about 1.4 to 10 per million. In The Netherlands it is proposed that for workers, a cancer risk

per compound of one extra cancer case per million a year should be striven for ("target risk") and that no risk higher than 100 per million a year ("prohibitory risk") should be tolerated. From the animal and the patient study it appears that the target risk is exceeded but that the risk is still below the prohibitory risk.

Introduction

Antineoplastic agents are widely used in the treatment of cancer and some non-neoplastic diseases [5,6]. Depending on their mechanism of action, these drugs are subdivided into several categories, e.g., alkylating agents, antimetabolites, mitotic inhibitors, and antibiotics. The alkylating agents express their cytotoxicity by interaction with the DNA of tumor cells. Because the desired cytotoxicity is not specific for cancer cells, normal cells may also be damaged, resulting in toxic side-effects. Carcinogenicity of a number of antineoplastic agents has been observed in animal studies and primary and secondary tumors have been found in patients treated with these drugs [4,10,11,13-17,21]. Cyclophosphamide (CP) is one of the most frequently used alkylating antineoplastic agents for different types of tumors [5,31]. According to the International Agency for Research on Cancer, there is sufficient evidence of carcinogenicity in humans and animals [13,14]. Due to its reactivity with DNA and mutagenicity in various short-term tests, CP is classified as a genotoxic carcinogen.

Because of the carcinogenic potency of CP and other antineoplastic agents, health care workers involved in the preparation and administration of these drugs may be at risk [30]. Therefore exposure to these compounds should be avoided, and safety guidelines and protective measures such as wearing masks, gloves, gowns, and special clothes and using laminar air-flow safety hoods have been introduced to protect workers when handling these drugs [1,29]. Nevertheless, in several studies it has been shown that uptake of CP does occur during occupational activities [24,25,27,28]. With a sensitive gas chromatographic-mass spectrometric method CP has been detected in the urine of health care workers handling antineoplastic drugs [26].

The risk of exposure to CP and other antineoplastic agents has been discussed in many studies [7,9,32]. However, a quantitative risk assessment could not be made because of the lack of the necessary exposure data. In this study we used data on the urinary excretion of CP in health care workers to estimate the uptake of CP. Next, a cancer risk assessment was carried out following two approaches based on (1) data from an animal study and (2) data on primary and secondary tumors in CP-treated patients.

Although we realize that risk assessment of occupational exposure to carcinogens is a complex matter, we think that such data are necessary to set

priorities in the protection of workers against genotoxic carcinogens In this paper we present an anitial approach to cancer risk estimation for health care workers exposed to CP The pitfalls are discussed

Materials and methods

Carcinogenicity studies in animals

The carcinogenicity of CP has been observed in several animal studies [13,14,21] For cancer risk assessment, studies are needed in which the animals are chronically exposed to at least two doses, preferably giving a clear dose-response relationship Only the study of Schmahl and Habs appears to satisfy this requirement [20] In this study male and female Sprague-Dawley rats were treated with CP in drinking water The treatment started when the rats were 100 days old Doses of 0, 0 31, 0 63, 1 25 and 2 5 mg/kg body weight were administered, 5 times a week, during their lifetime Forty rats were used for each sex and dose After a lifetime observation period, tumors of the urinary bladder and of the lymphoid and hematopoietic tissues (leukemias) were most frequently found and a dose-response relationship was obtained (Table 1) A statistically significant increase in leukemias was observed in males and females combined Only in males was a statistically significant increase in tumors of the urinary bladder found

Epidemiological studies in patients

Many cases of cancer have been reported after therapeutic treatment with CP [4,10,11,13,14,16,17,21] Among the various types of cancer, leukemias and tumors of the urinary bladder have been found most frequently not only as secondary tumors following cancer treatment but also as primary tumors after treatment for other non-neoplastic diseases In some of these studies the doses of CP were known and consequently the relative risks could be calculated [11,17] The results of these studies showed a dose-dependent increase in the relative risk of developing leukemias after treatment with CP for breast and ovarian cancer For cancer risk assessment only data can be used from studies in which, in addition to the dose of CP administered, the tumor incidence is given Both kinds of data were available, from two studies and these are briefly described below [4,10]

In five clinical trials the development of secondary leukemias was studied in 333 ovarian cancer patients treated with CP [10] For the total group, a 10 year cumulative incidence of 5 4% was calculated When the total group was subdivided into three dose groups, only in the highest dose group were leukemias observed, with a 10-year cumulative incidence of 11 1% For 1657 ovarian cancer patients not treated with CP a 10-year cumulative incidence of 0 1% was found

Table 1. Urinary bladder tumors and leukemias from oral cyclophosphamide treatment in male and female Sprague-Dawley rats[a].

dose 5 times a week (mg/kg body weight)	number of urinary bladder tumors		number of leukemias
	males	females	males and females
0	0/38	0/34	0/72
0.31	2/34	0/37	3/71
0.63	2/36	0/37	6/73[b]
1.25	5/35[c]	0/33	6/68[d]
2.5	7/31[e]	1/27	4/58[f]

[a] from Schmahl and Habs [20]
[b] Fischer exact test: $P=0.015$
[c] Fischer exact test: $P=0.022$
[d] Fischer exact test: $P=0.011$
[e] Fischer exact test: $P=0.0024$
[f] Fischer exact test: $P=0.037$

The development of tumors was also studied in patients with rheumatoid arthritis treated with CP as an immunosuppressive agent [4]. A total of 119 patients (76 women) were treated with a mean CP dose of 52.9 g while 119 matched controls received no CP. Tumors were observed in 24.4% of the CP-treated patients during an observation period of 10.8 year. A tumor incidence of 13.4% was observed in the matched controls during an observation period of 11.8 year. An increased incidence was observed for urinary bladder tumors (6 vs 0), skin cancer (8 vs 0) and leukemias (5 vs 1). The proportion of patients with tumors was significantly higher among men than women in the CP-treated group and in the control group. The increase in tumors between the control group and the CP-treated group was the same for men and women. Unfortunately, no information was available upon sex-specific tumor types.

Estimation of the daily uptake

Despite the introduction of safety guidelines and protective measures, in several studies it has been shown that (health care) workers involved in the preparation and administration of antineoplastic agents like CP are exposed to this particular compound, since uptake was established by detection of CP in urine (Table 2) [8,12,24,25,27,28]. In order to avoid the use of unreliable data obtained from urinary CP excretion after a rather short period of only a few days, urine of eight hospital pharmacy workers was collected during two to

four periods of 4 successive days. A total of 476 urine samples were analysed. CP was detected in one or more urine samples of each worker. The mean daily excretion was 0.18 μg (range: 0.01-0.53 μg). These urinary excretion levels are the basis for the estimation of the exposure. In urine samples with undetectable amounts of CP the amounts excreted were set at zero.

Table 2. Range of cyclophosphamide in urine of (health care) workers exposed to antineoplastic agents.

group	no. of workers	period of urine sampling (days)	mean amount[a] (range) of CP in urine (μg/day)	reference
health care workers	20	4	0.39 (0-2.5)	Evelo et al. [8]
nurses	2	57[b]	0.47 (0.43-0.51)[c]	Hirst et al. [12]
pharmacy technicians	2	2	0	Sessink et al. [23]
pharmacy technicians	25	1-2	0.05 (0-0.5)	Sessink et al. [24]
animal caretakers	4	2-5	0.05 (0-0.2)	Sessink et al. [25]
pharmacy technicians[d]	9	1-2	1.36 (0-10.05)	Sessink et al. [27]
pharmacy technicians[d]	9	5	0.16 (0-0.51)	unpublished
nurses	8	1	0.79 (0-2.9)	Sessink et al. [28]
pharmacy technicians	1			
cleaning women	2			
nurses	7	2-4	0.80 (0-4.2)	unpublished
pharmacy technicians	8	8-16	0.18 (0 01-0.53)	this study

[a] amounts below the detection limits were set at zero
[b] in both nurses urine sampling was performed over a total of 57 days
[c] mean and range were calculated by assuming a mean sampling period of 28½ days
[d] the same persons

It is supposed that during occupational activities antineoplastic agents like CP are absorbed by inhalation and via dermal penetration [2,12,18,27]. Suchlike exposure conditions were imitated in rats by intratracheal instillation and dermal application of a single CP dose of 1 mg/kg body weight [22]. For both treatments a total of about 5% of the applied dose was excreted unchanged in urine, all within 24 h. Application of 1 mg CP on the skin of volunteers revealed an urinary excretion of about 1% [12]. The mean uptake of CP was calculated by multiplication of the mean daily urinary CP excretion of 0.18 μg by 20 (5% excretion after dermal and intratracheal treatment of rats) or 100 (1% excretion after dermal application in volunteers). Hence, it was estimated that the mean daily uptake of CP will range from 3.6 to 18 μg.

Cancer risk assessment based on the animal study

Cancer risks were estimated using linear extrapolation and assuming that the time dependence of tumor development is more likely related to the cumulative dose of CP than to life span and the rate of aging [19]. The model is based on intersection of a straight line between zero dose and the lowest dose in the animal experiment at which a significant increase in tumors is observed (Table 1). From the results of the study of Schmahl and Habs, the median survival periods of the rats at the different doses were obtained [20]. To calculate the exposure periods, the survival periods were reduced by 100 days (at the start of the experiment the rats were 100 days old) and multiplied with 5/7 (the rats were administered CP 5 times a week) The total cumulative CP doses were calculated by multiplication of the doses by the exposure periods. The cancer risks per mg CP were calculated by dividing the tumor incidences by the total cumulative CP doses. Finally, lifetime cancer risks for health care workers were calculated by multiplication of the cancer risks per mg CP in rats by the total cumulative CP uptake of the health care workers over a working (exposure) period of 40 years (range: 28 8-144 mg) and the results were divided by body weight. An overview of the calculated data is given in Table 3

Cancer risk assessment based on the epidemiological studies

From the study of Greene et al., the 10-year cumulative cancer risk per gram was calculated by using the median dose of 19.5 g among the total group and the corresponding 10-year cumulative cancer incidence of 5.4% [10]. A linear dose-response curve was constructed, using the 10-year cumulative cancer risk of 0.1% which was observed in the control group of patients without CP treatment. Finally, a 10-year cumulative cancer incidence of 0.27% per gram was calculated. A 10-year cumulative cancer incidence of 0.24% per gram was calculated by using the data of the highest dose group of 46.35 g and the corresponding 10-year cumulative cancer incidence of 11 1%.

The same procedure was followed for the results of the study by Baker et al. [4] The 10-year cumulative cancer risk difference between CP-treated patients and control patients was calculated by linear extrapolation from the measured 10.8 year observation period to a 10 year period. The mean dose was 52.9 g, resulting in a 10-year cumulative cancer incidence of 0.21% per g.

Results

Cancer risk assessment based on the animal study

Cancer risks were calculated for a health care worker of 70 kg body weight and a working (exposure) period of 40 years, 200 days a year. Within the range of the estimated mean daily CP uptake of 3.6-18 μg (total cumulative CP uptake over 40 years: 28.8-144 mg), the calculated lifetime risks of urinary bladder cancer in men and leukemias in men and women were nearly the same and range from 120 to 600 per million and 95 to 475 per million, respectively (Table 3).

Table 3. Cancer risk calculations for health care workers obtained by linear extrapolation using incidence of tumor of the urinary bladder in male rats and leukemias in male and female rats[a].

parameter	urinary bladder tumors	leukemias
rat		
lowest dose with significant increase in tumors[b] (mg CP/kg body weight per day)	1.25	0.63
tumor incidence[b] (%)	14.3	8.2
median survival period (days)	646	889
exposure period (days)	(646-100)x5/7 = 390	(889-100)x5/7 = 564
total cumulated dose (mg CP/kg body weight)	1.25x390 = 487.5	0.63x564 = 355
cancer risk per mg CP (kg body weight/mg CP)	$0.143/487.5 = 293 \times 10^{-6}$	$0.082/355 = 233 \times 10^{-6}$
health care worker		
lifetime cancer risk[c] (per million)	1/70x293x(28.8-144) = 120-600	1/70x233x(28.8-144) = 95-475

[a] from Schmahl and Habs [20]
[b] from Table 1
[c] 70 kg body weight; total cumulative CP uptake over 40 years: 28.8-144 mg

Cancer risk assessment based on epidemiological studies

Cancer risks were calculated by multiplication of the 10-year cumulative incidence per gram CP in patients by the estimated mean total CP uptake in health care workers over 10 years, 200 days a year, based on the estimated mean daily uptake of 3.6-18 μg (total cumulative CP uptake over 10 years: 7.2-36 mg). No differences were found in the study on secondary tumors by Greene et al. between the results based on calculations with the median dose of the total group of 19.5 g and the highest dose group of 46.35 g (Table 4) [10]. In both cases cancer risk ranged from about 17 to 100 per million. A marginally lower cancer risk of 15 to 76 per million was obtained when primary tumor data from the study by Baker et al. were used (Table 4) [4].

Table 4. Cancer risks for health care workers obtained from tumor incidence data of patients.

patients[a]		exposed workers[b]	reference
CP dose (g)	cancer risk (per million)	cancer risk (per million)	
leukemias in women			
19.5[c]	54000	20-100	Greene et al. [10]
46.35[d]	111000	17-86	
0[e]	1000		
urinary bladder tumors and leukemias in men and women			
52.9[f]	112000	15-76	Baker at al. [4]

[a] 10-year observation period
[b] 10-year exposure period (total cumulative CP uptake over 10 years: 7.2-36 mg)
[c] median dose
[d] highest dose group
[e] control group without CP treatment
[f] mean dose

Discussion

In the present study a cancer risk assessment of occupational exposure to CP was carried out following two approaches based on (1) data from an animal study and (2) data based on primary and secondary tumors in CP-treated patients During this operation several assumptions were made, and these are discussed below

Tumor type

The same tumor types, namely urinary bladder tumors and leukemias, were found in the animals and in the patients It is remarkable that urinary bladder tumors only appeared in male rats while leukemias were observed equally in male and female rats Unfortunately, no information is available from the patient study of Baker et al with respect to the sex-specific induction of urinary bladder tumors [4] Consequently it is assumed that no sex differences were present Because the study of Greene et al only included women, it remains impossible to establish whether leukemias were induced in a sex-specific manner [10] For extrapolation of the results of the animal study and patient studies this means that (1) the animal study may only be used for risk assessment of urinary bladder tumors in men and leukemias in men and women, (2) extrapolation from the patientstudy of Greene et al is restricted to risk assessment of leukemias in women, and (3) it is assumed that the patient study of Baker et al is suitable for the risk assessment of urinary bladder tumors and leukemias in men and women [4,10]

Estimation of the daily uptake

For the estimation of the mean daily CP uptake, the excretion of unmetabolized CP in urine was investigated In order to obtain accurate information about the daily CP excretion in urine, urine samples were collected from several persons over two to four periods of 4 successive days In this way incidentally extremely high levels were averaged and overestimation was minimized Nevertheless, a large interindividual difference in CP excretion was obtained, undoubtedly due to interindividual differences in uptake and/or biotransformation of CP In order to compare the results of the different studies in a proper way, undetectable amounts of CP were set at zero This results in an underestimation of the cancer risk For the estimation of the uptake, we used results of studies in which relatively low amounts of CP were administered to rats and volunteers Data of patients were not used because the amounts of CP administered were much higher than would be expected after occupational exposure

The results of the animal study showed that a total of about 5% of a single CP dose of 1 mg/kg body weight was excreted in urine, all within 24 h, irrespective of the route of exposure (intratracheal, dermal, oral, intravenous)

[22] Lowering of the dermally applied dose to 0.1 and 0 01 mg/kg body weight resulted in excretion percentages of 8 3% ± 2 1% and 5 1% ± 2.9%, respectively (mean ± S D., $n=5$); these figures are not significantly different from one another (unpublished results) Therefore the excretion of unmetabolized CP is estimated to be about 5% of the administered dose It is unclear why the percentage recovery of CP in urine in the volunteer study was only about 1% [12]. This may, of course, have been due to interspecies differences, but on the other hand it may also have been the result of incomplete absorption by the human skin. This latter possibility, which seems quite reasonable, would lead to an overestimation of both mean daily uptake and cancer risk if 1% excretion is taken as a starting point. Nevertheless, this is the only study in which volunteers have been treated with a relatively low CP dose. It should also be pointed out that the influence of chronic low-dose exposures by different exposure routes on the excretion percentage remains unknown.

Extrapolation

The data from the animal study showed a linear dose response curve between daily CP dose and cancer risk Consequently, linear extrapolation was used for cancer risk assessment It should be noted that cancer risk is not primarily caused by the amount of CP absorbed but depends on the amount of phosphoramide mustard formed after biotransformation [31]. The formation of phosphoramide mustard results in the alkylation of target DNA and cancer risk is expected to correlate better with the amount of phosphoramide mustard able to bind to target tissue. On the basis of the linear dose-response relationship in the animal study between CP dose and cancer risk we assume that the amount of phosphoramide mustard increases linearly with the CP dose. Since biotransformation of CP in man and rat are similar, it is also reasonable to assume that in patients the amount of phosphoramide mustard will increase linearly with the dose [13,31] This explains our choice of the linear extrapolation when using the patient data

Cancer risk

The results of the animal study showed a lifetime risk (70 years) of urinary bladder tumors in men and of leukemias in men and women of 95-600 per million after a period of CP exposure of 40 years, 200 days a year. Comparable cancer risks of 1.5-10 per million a year were estimated from the patient studies. It should be noted that in the animal study the number of lifetime cases was indicated while the cancer risk in patients was estimated on an observation period of 10 years. Since it is unclear how the dose-response curve will be after the 10-year observation period, it is not possible to show what the consequences will be for lifetime cancer risks Therefore the calculated risks most probably are underestimates. It should also be noted that

in the patient study of Greene et al. a possible predisposition of the patients under consideration may have resulted in higher cancer incidences upon treatment with genotoxic carcinogens [10]. It should be emphasized that in the study of Baker et al. the total cancer risk was assessed [4]. Not only urinary bladder tumors and leukemias were considered but also other types of cancer, especially skin cancer. This may partly explain the lower cancer risk calculated from the animal study as only urinary bladder tumors and leukemias could be taken into account here. Risks based on animal data should be calculated from the number of tumor-bearing animals, i.e., the sum of the number of animals having tumors at sites that show a statistically increased incidence as compared to controls. Unfortunately, the data of Schmähl and Habs do not allow such an approach, which might have resulted in higher estimated cancer risks [20].

Cancer risk and legislation

In the United States, Sweden, Germany, and the European Union, treshold limit values or comparable maximum exposure levels have been introduced for some (genotoxic) carcinogens in order to protect workers [3]. In The Netherlands it is proposed that, for workers, a cancer risk for each compound of 1 extra cancer case per million a year should be striven for ("target risk") and that no risk higher than 100 per million a year ("prohibitory risk") should be accepted [3]. From the animal and the patient study it appeared that the target risk is exceeded but that the risk is still below the prohibitory risk. Nevertheless, it should be noted that in some studies groups of workers have excreted higher amounts of CP than the mean daily CP excretion of 0.18 μg in the present study, consequently resulting in higher cancer risks (Table 2).

It should be emphasized that this risk assessment was based on exposure to one single antineoplastic agent while it is known that more antineoplastic agents are used for which no exposure data are available. However, it is reasonable to assume that exposure to all antineoplastic agents will occur in a similar manner because the drugs are mostly handled in a comparable way. In fact, this means that the cancer risk might be higher than assessed in the present study, supposing that no antagonistic interaction takes place. However, this is not of influence in possible surpassing of target and prohibitory risk levels because these are based on single compounds.

In conclusion: The calculated cancer risk due to occupational exposure to CP was based on available dose-response data and on the indirect assessment of CP exposure by measuring CP excretion in urine. Although the presented cancer risk assessment due to exposure to CP has its limitations, the cancer risks calculated from CP exposure of health care workers indicate that these workers still have higher risks for cancer due to the handling of CP and/or other antineoplastic agents in spite of the protective measures that have been taken.

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Chapter 6

General discussion and summary

The aim of the research studies presented in this thesis was the development and validation of methods for monitoring of occupational exposure to antineoplastic drugs. After evaluation of the methods used hitherto, it was concluded that there is a need for sensitive and compound-specific methods for EM and BM. Data about the use of parenterally administered antineoplastic drugs in the Netherlands over 1986-1988 showed that CP, IP, and 5FU were most frequently used. During the validation studies, it was observed that in addition to these drugs, MTX was also often administered. Therefore, CP, IP, 5FU, and MTX were selected for monitoring.

Development and validation of methods for environmental and biological monitoring of antineoplastic agents

Environmental monitoring

Methods for the determination of CP, IP, 5FU and MTX in environmental samples were developed and applied in several studies to measure the release and the spread out of these drugs during several occupational activities. In principle, the EM methods were developed to validate the BM methods.

For the determination of CP and IP in air- and wipe samples a sensitive GC-MS method was developed. 5FU and MTX were determined using HPLC methods. In general the drugs are extracted from the air- and wipe samples with a sodium hydroxide solution. In the case of 5FU and MTX, the extracts can almost directly be analysed. For the determination of CP and IP, the same extracts are further cleaned up by extraction with an organic solvent. The organic solvent is evaporated and the extract is derivatised with trifluoroacetic anhydride. The limit of detection for 5FU ranges from 7 to 65 ng/ml extract. For CP, IP and MTX, the limits of detection are 0.25, 0.25, and 0.60 ng/ml extract, respectively.

Exposure to CP, 5FU, and MTX was measured by the analysis of these compounds in air samples and wipe samples from possibly contaminated surfaces and objects.
During preparation of the drugs, stationary air samples were taken in front of

the laminar air-flow hoods. CP (0.2 $\mu g/m^3$) and MTX (0.3 $\mu g/m^3$) were detected once. Personal air samples were also taken. The CP concentration ranged up to 10.1 $\mu g/m^3$. Contamination of the work environment was found. On the working trays of the laminar air-flow hoods CP, 5FU, and MTX were present (CP: to 160 ng/cm^2; 5FU: to 62 ng/cm^2; MTX: to 633 ng/cm^2). Even after cleaning of the laminar air-flow hood, it remained contaminated (CP: to 14 ng/cm^2; 5FU: to 27 ng/cm^2; MTX: to 293 ng/cm^2). On the floors in front of and beneath the laminar air-flow hoods (CP: to 2.6 $\mu g/m^2$; 5FU: to 236 $\mu g/m^2$) and on the floors of the adjacent rooms (5FU: to 107 $\mu g/m^2$) the presence of CP and 5FU was established. The gloves used during preparation of the drugs (CP: to 149 μg/pair of gloves; 5FU: to 1070 μg/pair of gloves; MTX to 1963 μg/pair of gloves) and used during cleaning of the hoods (CP: to 11 μg/pair of gloves; 5FU: to 59 μg/pair of gloves; MTX: to 49 μg/pair of gloves) were frequently contaminated. Incidentally on vials (CP: 0.06 μg; MTX: 15 μg) used for the preparation of the drugs and on packings of prepared drugs (CP: 2.1 μg; 5FU: to 6.2 μg) CP, 5FU and MTX were detected.
The floors in the administration rooms, the floors in adjacent rooms (CP: 0.9 ng/cm^2; MTX: to 5.9 ng/cm^2) and the floors around the beds of the patients were contaminated with CP (median: 4.5 $\mu g/cm^2$) and 5FU (median: 1.8 ng/cm^2). Incidentally CP and 5FU were found on other objects such as tables (CP: to 4.5 μg; 5FU: to 22 μg) and cleaned urinals (CP: 8.3 μg).

Comparable results were found in a situation where animal keepers were involved in the injection of CP to laboratory animals. CP was detected on filters of the air-circulation system (to 1.0 μg/day) suggesting contamination of the environmental air although on the masks of the workers no CP was found. CP was detected in wipe samples taken from the floor (to 0.4 ng/cm^2), several objects and surfaces (to 44 ng/cm^2). CP was also detected in adjacent rooms (to 6.9 ng/cm^2). The gloves (to 199 μg) and sleeve protectors (to 10 μg) used during the injection were also contaminated with CP.

Although special guidelines and protective measures have been introduced to protect hospital workers during the handling of antineoplastic agents, it was found that they do not prevent the presence of these toxic compounds in the work environment. The introduction of additional protective measures such as adaptations of the laminar downflow hood, the use of special masks and double pairs of gloves by the workers and the replacement of 5FU ampules in 5FU vials resulted in a decrease of the concentrations of CP in the air during preparation of the drugs. It is suggested that replacement of 5FU ampules by 5FU vials is probably responsible for the diminished contamination of the latex gloves with 5FU. Possibly the breaking of ampules is a cause for the release of aerosols resulting in the contamination of the gloves. The results suggest that the introduced additional protective measures have reduced the external

exposure to CP and 5FU To what extent each of the additional protective measures has reduced the exposure, remains unknown

Environmental exposure to 5FU and MTX was measured in a pharmaceutical plant where workers were involved in the production of these drugs
5FU was found by the analysis of air and wipe samples During weighing, 5FU was detected in the air (75 $\mu g/m^3$) 5FU was also measured on the filter of the mask of the worker involved in weighing the drug (120 μg) Before drug compounding, 5FU was found on the floor (up to 8 ng/cm^2) After routine cleaning significantly higher amounts of 5FU were measured (70-630 ng/cm^2) suggesting ineffective cleaning procedures The gloves used were always contaminated (22-720 μg/pair of gloves)
MTX was detected in air samples of all workers except for those involved in the vial filling process (0 8-182 $\mu g/m^3$) The highest concentrations were observed for workers weighing MTX (118 and 182 $\mu g/m^3$)
The results presented above show that the methods developed and used are applicable to measure the release and the spread out of CP, 5FU, and MTX after several occupational activities

Biological monitoring

A sensitive GC-MS method was developed for the determination of CP and IP in urine Sample preparation (liquid-liquid extraction) followed by derivatisation with trifluoroacetic anhydride makes the method appropriate for routine analysis and enables to measure the uptake of these drugs during the preparation and administration. The limit of detection is approximately 0 25 ng/ml urine.
In several studies presented in this thesis, the method was validated. The uptake of CP and IP was measured by determination of these drugs in the urine of workers such as pharmacists and pharmacy technicians involved in drug preparation, nurses involved in drug administration and animal keepers involved in the injection of chemicals to laboratory animals The highest CP excretion rate observed was about 10 μg/day (Table 1) However, it is shown that there were large differences between the groups It should be noted that the most reliable information about the magnitude of the urinary excretion rate of CP is obtained by analysing urine samples of a large group of workers for a long period. CP and IP were found not only in the urine of pharmacy technicians, nurses and an animal keeper actively handling these compounds but also in the urine of pharmacy technicians and nurses not directly involved in the preparation and administration of these drugs CP and IP were excreted during different periods after the beginning of the working period, suggesting different times of exposure, different exposure routes, and/or interindividual

differences in biotransformation and excretion rate for these compounds.

Table 1. Overview of several studies concerning the measurement of excretion rates of CP in urine in (health care) workers exposed to antineoplastic agents

group	number of workers	period of urine sampling (days) in urine	mean excretion rate[a] (range) of CP (μg/day)	reference
nurses	2	57[b]	0.47 (0.43-0.51)[c]	[1]
health care workers	20	4	0.39 (0-2.5)	[2]
pharmacy technicians	2	2	0	chapter 2.1
pharmacy technicians/nurses	18	1-2	0.05 (0-0.5)	chapter 2.2
animal keepers	4	2-5	0.05 (0-0.2)	chapter 2.4
pharmacy technicians[d]	9	1-2	1.44 (0-10)	chapter 2.5
pharmacy technicians[d]	9	5	0.16 (0-0.51)	chapter 2.6
nurses	8	1	0.79 (0-2.9)	chapter 3.1
pharmacy technicians	1			
cleaning women	2			
nurses	7	2-4	0.80 (0-4.2)	
pharmacy personnel/nurses	21	1-5	5.2 (0-38)	[3]
pharmacy technicians	8	8-16	0.18 (0.01-0.53)	chapter 5.1

[a] the excretion rates calculated for amounts below the detection limits were set zero
[b] both nurses sampled urine over a total of 57 days
[c] mean and range were calculated by assuming a mean sampling period of 28½ days
[d] the same persons

[1] Evelo CTA, Bos RP, Peters JGP, Henderson PTh. Urinary cyclophosphamide assay as a method for biological monitoring of occupational exposure to cyclophosphamide. Int Arch Occup Environ Health (1986) 58:151-155.
[2] Hirst M, Tse S, Mills DG, Levin L. Occupational exposure to cyclophosphamide. The Lancet (1984):186-188.
[3] Ensslin AS, Stoll Y, Pethran A, Pfaller A, Rommelt H, Fruhmann G. Biological monitoring of cyclophosphamide and ifosfamide in urine of hospital personnel occupationally exposed to cytostatic drugs. Occup Environ Med (1994b) 51:229-233.

Although special guidelines and protective measures have been introduced to protect hospital workers during the handling of antineoplastic agents, it was found that they do not prevent the uptake of these toxic compounds. Therefore, in one study additional protective measures were introduced to

investigate whether the developed methods could be used in intervention studies The measures included adaptations of the laminar downflow hood, the use of special masks and double pairs of gloves by the workers and the replacement of 5FU ampules by vials The effects of these additional measures were compared with the results of a previous study It was shown that the introduced additional protective measures reduced the external exposure to CP The mean CP excretion rate before and after the intervention was 1 44 and 0 16 μg/day, respectively The difference was not statistically different

For the determination of MTX in urine after occupational exposure, a FPIA method was developed FPIA, is frequently used for monitoring serum levels in patients treated with MTX The FPIA method was modified in such a way that MTX could be measured quickly and efficiently in urine samples of exposed workers The urine samples are purified using solid phase extraction Concentrations of at least 1 equivalent MTX/ml urine could be detected
A GC-MS method was developed for the determination of FBAL, a main metabolite of 5FU in urine of exposed workers Before analysis, FBAL is derivatized with S-ethyltrifluorothioacetate and acidified n-butyl alcohol The detection limit is about 60 ng/ml urine
The methods developed for biological monitoring of exposure to 5FU and MTX have been validated in a pharmaceutical plant where workers are involved in the production of 5FU and MTX Fifty micrograms of FBAL were found in one urine sample of a worker weighing 5FU The excretion rate of MTX over 72-96h was higher for the exposed groups of workers (mean 4 2 μg MTX-equivalents/24 h, range 1 5-7 0 μg MTX-equivalents/24 h) compared to their controls (mean· 3 2 μg MTX-equivalents/24 h, range 1 5-6 6 μg MTX-equivalents/24 h)

The developed methods have been proven to be appropriate for biological monitoring of occupational exposure to CP, IP, 5FU and MTX The methods have been applied in several studies It appeared that the methods were appropriate for the measurement of the uptake of the compounds in the body of the workers The results of several studies described here have shown that during the preparation and administration of antineoplastic agents, among which CP, the workers were internally exposed to CP Uptake of CP was even found in pharmacy technicians involved in the preparation of antineoplastic drugs other than CP The exact exposure routes could not be identified although it was indicated that inhalation was probably not the only one Especially the results of hospital workers, with CP in their urine, not preparing or administering CP, shows the surplus value of this BM method

Biological effects in workers exposed to antineoplastic agents

To study a possible relationship between uptake and an early biological response in hospital workers, the excretion of CP in urine and the presence of chromosomal aberrations in peripheral blood lymphocytes was determined in four groups of subjects with various exposure statuses (17 Dutch and 11 Czech exposed hospital workers handling antineoplastic agents and 35 Dutch and 23 Czech control hospital workers not handling these drugs) The groups were subdivided into smokers and non-smokers.
Within the Dutch groups, the percentage of aberrant cells (3 2 versus 2 0) and the number of breaks per cel (0 042 versus 0 023) were increased for smokers compared to non-smokers The percentage of aberrant cells was increased in Dutch exposed workers in comparison with Dutch control workers (2 6 versus 2 1) Within the Czech groups the percentage of aberrant cells (2 7 versus 1 8) and the number of breaks per cel (0 034 versus 0 020) were increased in exposed workers in comparison with control workers However, both Dutch and Czech smokers mainly contributed to the increase The results suggest an additive effect of exposure and smoking in the Dutch subjects and a more than additive effect in the Czech subjects
In the urine samples of 3 out of 11 Dutch exposed workers the CP excretion rate ranged from 0 1 to 0 5 μg/24 h Higher excretion rates were found for 8 out of 11 Czech exposed workers, ranging from 0 1 to 2 9 μg/24 h No correlation was observed between the amounts of CP excreted in urine on the one hand and the percentage of aberrant cells and the number of breaks per cell on the other hand
The results suggest that urinary levels of CP up to 2 9 μg/24 h may result in biological effects like chromosomal aberrations in the long run Since chromosomal aberrations are considered to be indicators of an increased genetic risk, these urinary CP concentrations may not only demonstrate absorption of CP but may also indicate a serious risk, especially for smokers

In order to study nephrotoxic effects due to occupational exposure to antineoplastic agents, the early renal effect parameters retinol-binding protein (RBP) and albumin (ALB) in urine have been determined in 11 hospital workers involved in the preparation and administration of antineoplastic agents A group of 23 hospital workers not involved in drug handling served as non-exposed controls No difference was found between the exposed group and the non-exposed control group with respect to RBP (7 9 μg/mol CREAT-U versus 8 8 μg/mol CREAT-U) and ALB (0 44 mg/mol CREAT-U versus 0 30 mg/mol CREAT-U) Although it was demonstrated that the hospital workers were exposed to CP, measured by the urinary CP excretion rate (0 1-2 9 μg/24 h), the results of the present study show that these exposure levels did not cause any defined nephrotoxic effects

Toxicokinetic studies on cyclophosphamide (uptake, biotransformation, and excretion)

Inhalation and absorption through the skin are supposed to be the most important routes of occupational exposure to cytostatic drugs. In a study, the excretion rate of unmetabolized CP in rat urine was investigated after intratracheal instillation and dermal application, mimicking possible occupational exposure routes. The results show that the total amount of CP excreted (about 5% of the dose was excreted unchanged) was independent of the exposure route. The lowest excretion rate was observed after dermal application.
Our results in rats, along with those of Hirst et al. in dermal-treated volunteers, demonstrate that CP easily penetrates rat and human skin [1]. Therefore, dermal exposure must be considered as a possible exposure route for CP.

The in vitro biotransformation of CP was studied with S-9 liver fractions of human donors. Thereto, sensitive methods for the determination of the CP metabolites NNM, KCP and CAR were developed. After liquid-liquid extraction and derivatization, the metabolites are determined using gas chromatography and thermionic specific detection. The results show large interindividual differences in the formation of NNM and CAR. KCP was not detected.

The biotransformation of CP was also investigated in the rat in vitro with S9 liver fractions. The aim was to investigate a possible influence of the environmental factors smoking and alcohol consumption on the biotransformation of CP. The effects were mimicked by pretreatment of rats with ß-naphthoflavone and ethanol, respectively. The influence of sex and supplementation of the incubates with NAD and GSH was also studied. The in vitro hepatic biotransformation of CP in rat was strongly influenced by sex and by supplementation with NAD and GSH. No influence of pretreatment with ß-naphthoflavone and ethanol was observed. The results suggest that the influence of the environmental factors smoking and alcohol consumption on the biotransformation of CP in man will be negligible.

Risk assessment of occupational exposure to cyclophosphamide

A cancer risk assessment of occupational exposure to the genotoxic carcinogenic antineoplastic agent CP was carried out following two approaches (1) based on data from an animal study, and (2) based on data of primary and secondary tumors in CP-treated patients. The uptake of CP was

estimated based on the urinary excretion of CP in several workers exposed to antineoplastic agents such as CP The uptake varies from 3 6-18 μg per day Cancer risk was estimated on the basis of dose-response data from an animal study and data from patient studies Several assumptions were made Cancer risk based on the animal study was calculated for a 70 kg worker and an exposure period of 40 years, 200 days a year Lifetime risk of bladder cancer in men and leukemias in women were approximately the same and vary from 100-600 per million at a daily dose of 3 6-18 μg Cancer risk based on the patient study was calculated by multiplication of the 10 year cumulative incidence per g CP with the mean daily uptake over 10 years, 200 days a year ranging from 7 2 to 36 mg Based on secondary tumor data the risk of leukemias in women was calculated to be 17-100 per million over 10 years A marginally lower risk for bladder cancer and leukemias in men and women of 15-76 was found when primary tumor data were used Thus, on an annual basis, cancer risks obtained from both the animal and the patient study were nearly the same and ranged from about 1 4 to 10 per million

In The Netherlands it is proposed that, for workers, a cancer risk for each compound of 1 extra cancer case per million a year should be striven for ("target risk") and that no risk higher than 100 per million a year ("prohibitory risk") should be accepted From the animal and the patient study it appeared that the target risk is exceeded but that the risk is still below the prohibitory risk Nevertheless, it should be noted that in some studies groups of workers have higher CP excretion rates than the mean daily CP excretion rate of 0 18 μg used for the present cancer risk assessment (Table 1) For example, in a comparable study with 7 nurses a mean excretion rate of 0 80 μg/day was found Consequently, this about 5 times higher CP excretion rate may result in a correspondingly higher cancer risk It should be noted that the present risk assessment only concerns cancer

Despite the introduction of "target risk" and "prohibitory risk" levels, it should be emphasized that it is the task of employees, employers, and authorities to avoid every case of cancer due to occupational exposure to CP and other antineoplastic drugs

In conclusion

The methods described in this thesis may offer an important contribution to trace occupational exposure to antineoplastic agents and consequently to reduce the possible health-risks of the workers involved in handling these drugs

Samenvatting

Het doel van het onderzoek dat in dit proefschrift getiteld **Monitoring van beroepsmatige blootstelling aan cytostatica** wordt beschreven, is het ontwikkelen en valideren van methoden voor het meten van beroepsmatige blootstelling aan cytostatica. Uit de beoordeling van de methoden die tot het begin van het in dit proefschrift beschreven onderzoek in gebruik waren bleek dat gevoelige en specifieke methoden voor omgevingsmonitoring en voor biologische monitoring van beroepsmatige blootstelling aan cytostatica in feite niet voorhanden waren. Uit gegevens over het gebruik van parenteraal toegediende cytostatica in de Nederlandse ziekenhuizen gedurende 1986-1988 bleek dat cyclofosfamide (CP), ifosfamide (IP) en 5-fluorouracil (5FU) het meest frequent werden gebruikt. Tijdens het onderzoek bleek dat methotrexaat (MTX) eveneens veelvuldig werd toegediend. Om deze reden zijn CP, IP, 5FU en MTX geselecteerd als modelstoffen voor het ontwikkelen van monitoringsmethoden.

Ontwikkeling en validering van methoden voor omgevingsmonitoring en biologische monitoring van cytostatica

Omgevingsmonitoring

Methoden voor het bepalen van CP, IP, 5FU en MTX in omgevingsmonsters zijn ontwikkeld en in verschillende studies toegepast, met als doel het vrijkomen en de verspreiding van deze stoffen gedurende verschillende beroepsmatige activiteiten te kunnen meten. De methoden voor omgevingsmonitoring zijn vooral ontwikkeld om de methoden voor biologische monitoring te kunnen valideren.

Voor het bepalen van CP en IP in lucht- en veegmonsters is een gevoelige GC-MS methode ontwikkeld. 5FU en MTX worden geanalyseerd met behulp van HPLC. Allereerst worden de stoffen uit de lucht- en veegmonsters met een basische oplossing geëxtraheerd. Voor het bepalen van 5FU en MTX worden de extracten vervolgens geanalyseerd. Voor het bepalen van CP en IP worden dezelfde extracten verder opgewerkt door een extractie met een organisch oplosmiddel. Het oplosmiddel wordt ingedampt waarna het extract wordt gederivatiseerd met trifluorazijnzuuranhydride. De detectiegrens varieert van 7 tot 65 ng/ml extract voor 5FU. Voor CP, IP en MTX zijn de detectiegrenzen achtereenvolgens 0,25, 0,25, en 0,60 ng/ml extract.

De blootstelling aan CP, 5FU en MTX werd gemeten door het analyseren van deze stoffen in lucht- en veegmonsters van mogelijk gecontamineerde oppervlakken en voorwerpen.
Gedurende de bereiding van de cytostatica in de apotheek van een ziekenhuis zijn stationaire luchtmonsters genomen voor de laminar airflow (laf)-kast. CP (0,2 $\mu g/m^3$) en MTX (0,3 $\mu g/m^3$) zijn éénmaal gedetecteerd. Daarnaast werden persoonsgebonden luchtmonsters verzameld. De CP concentratie bedroeg maximaal 10,1 $\mu g/m^3$. Contaminatie van de werkomgeving werd eveneens waargenomen. Op het werkblad van de laf-kast werden CP, 5FU en MTX aangetoond (CP: maximaal 160 ng/cm^2, 5FU: maximaal 62 ng/cm^2; MTX: maximaal 633 ng/cm^2). Zelfs na het schoonmaken van de laf-kast bleef de contaminatie aanwezig (CP· maximaal 14 ng/cm^2, 5FU: maximaal 27 ng/cm^2; MTX. maximaal 293 ng/cm^2). Op de vloeren voor en onder de laf-kast (CP· maximaal 2,6 $\mu g/m^2$; 5FU: maximaal 236 $\mu g/m^2$) en op de vloeren van de aanliggende ruimten (5FU· maximaal 107 $\mu g/m^2$) werden CP en 5FU aangetoond De handschoenen die tijdens de bereiding van de cytostatica (CP: maximaal 149 μg/paar handschoenen; 5FU· maximaal 1070 μg/paar handschoenen; MTX maximaal 1963 μg/paar handschoenen) en gedurende het schoonmaken van de laf-kasten (CP· maximaal 11 μg/paar handschoenen; 5FU maximaal 59 μg/paar handschoenen; MTX: maximaal 49 μg/paar handschoenen) werden gebruikt, waren meestal gecontamineerd. Af en toe werd contaminatie van de buitenzijde van de flacons, die werden gebruikt voor de bereiding van de cytostatica, aangetoond (CP: 0,06 μg; MTX: 15 μg) evenals contaminatie van de verpakking van de bereide cytostatica (CP· 2,1 μg, 5FU: maximaal 6,2 μg)
De vloeren van de toedieningsruimten en de aanliggende ruimten (CP: 0,9 ng/cm^2, MTX maximaal 5,9 ng/cm^2) en rondom de bedden van de patienten waren eveneens gecontamineerd met CP (mediaan: 4,5 $\mu g/cm^2$) en 5FU (mediaan: 1,8 ng/cm^2). Af en toe werd CP en 5FU op andere voorwerpen aangetoond zoals tafels (CP: maximaal 4,5 μg; 5FU: maximaal 22 μg) en omgespoelde urinalen (CP: 8,3 μg).

Vergelijkbare resultaten werden geconstateerd in een situatie van proefdierverzorgers die betrokken waren bij het injecteren van CP bij proefdieren. CP werd gedetecteerd op de filters van het luchtventilatiesysteem (maximaal 1,0 μg/dag) dat contaminatie van de omgevingslucht suggereerde alhoewel op het mondmasker van de proefdierverzorgers geen CP werd gevonden. CP werd ook gedetecteerd in veegmonsters, die van de vloer zijn genomen (maximaal 0,4 ng/cm^2), evenals van verschillende voorwerpen en oppervlakken (maximaal 44 ng/cm^2) CP werd ook gedetecteerd in de aanliggende ruimten (maximaal 6,9 ng/cm^2) De handschoenen (maximaal 199 μg) en mouwbeschermers (maximaal 10 μg) die tijdens de injecties werden gedragen, bleken ook gecontamineerd met CP te zijn.

Alhoewel speciale richtlijnen en beschermende maatregelen voor het omgaan van ziekenhuispersoneel met cytostatica zijn geintroduceerd, is aangetoond dat deze de aanwezigheid van dergelijke toxische stoffen in de werkomgeving niet uitsluiten De introductie van aanvullende beschermende maatregelen zoals aanpassingen aan de laf-kast, het gebruik van speciale maskers, het dragen van twee paar handschoenen over elkaar en het vervangen van 5FU in ampullen door 5FU in flacons resulteerde in een afname van de CP concentratie in de lucht tijdens de bereiding van de cytostatica Verondersteld wordt dat de vervanging van ampullen met 5FU door flacons met 5FU waarschijnlijk de reden is voor de verminderde contaminatie van latex handschoenen met 5FU Mogelijk is het breken van ampullen de oorzaak van het vrijkomen van aerosolen dat resulteert in contaminatie van de handschoenen De resultaten suggereren dat de aanvullende beschermende maatregelen de externe blootstelling aan CP en 5FU hebben gereduceerd In welke mate de aanvullende beschermende maatregelen elk afzonderlijk de blootstelling hebben beinvloed, blijft onbekend.

De blootstelling aan 5FU en MTX werd gemeten in een farmaceutisch bedrijf waar werknemers betrokken waren bij de formulering van deze cytostatica (wegen, mengen, filtreren en afvullen)
5FU werd aangetoond in lucht- en veegmonsters 5FU werd gedurende het afwegen in een luchtmonster gedetecteerd (75 $\mu g/m^3$) 5FU is eveneens aangetoond op het filter van het masker van de medewerker die betrokken was bij het afwegen van de stof (120 μg) Voorafgaand aan het produktieproces werd 5FU op de vloer van de werkruimte aangetoond (maximaal 8 ng/cm^2) Na de routinematige schoonmaak van de ruimte werden significant grotere hoeveelheden gemeten (70-630 ng/cm^2) De resultaten suggereren een ineffectieve schoonmaakprocedure De handschoenen waren altijd gecontamineerd (22-720 μg/paar handschoenen).
MTX is gedetecteerd in luchtmonsters in de ademzone van alle medewerkers met uitzondering van diegenen die betrokken waren bij het vullen van de flacons bij de afvulmachine (0,8-182 $\mu g/m^3$) De hoogste concentraties werden waargenomen bij de medewerkers die MTX afwogen (118 and 182 $\mu g/m^3$)
Bovenstaande resultaten tonen aan dat de ontwikkelde methoden bruikbaar zijn om het vrijkomen en de verspreiding van CP, 5FU en MTX bij verschillende beroepsmatige activiteiten te meten

Biologische monitoring

Er werd een gevoelige GC-MS methode ontwikkeld voor het bepalen van CP en IP in urine De monstervoorbereiding (vloeistof-vloeistof extractie) gevolgd door derivatisering met trifluorazijnzuuranhydride maakt de methode geschikt

voor routinematige analyse en maakt het mogelijk de uitscheiding van deze cytostatica in de urine van ziekenhuispersoneel betrokken bij de bereiding en de toediening van cytostatica te meten De detectiegrens is ongeveer 0,25 ng/ml urine en kan worden verlaagd tot 0,1 ng/ml door gebruik te maken van GC-MSMS
In verschillende studies die in dit proefschrift worden beschreven is de methode gevalideerd De opname van CP en IP is gemeten door het bepalen van deze cytostatica in de urine van de medewerkers zoals apothekers (assistenten) betrokken bij de bereiding van de cytostatica, verpleegkundigen betrokken bij de toediening van de cytostatica en proefdierverzorgers betrokken bij de toediening van CP aan proefdieren De hoogste CP uitscheidingssnelheid bedroeg ongeveer 10 μg/dag (Tabel 1) Er werd echter aangetoond dat er grote verschillen waren tussen de groepen Hierbij dient te worden opgemerkt dat de meest betrouwbare informatie over de grootte van de uitscheidingssnelheid van CP in de urine wordt verkregen door urinemonsters van een grote groep personen verzameld over een lange periode te analyseren CP en IP werden niet alleen aangetoond in de urine van apothekersassistenten, verpleegkundigen en proefdierverzorgers die daadwerkelijk met de CP en IP omgingen, maar ook in de urine van apothekersassistenten en verpleegkundigen die niet betrokken waren bij de bereiding en de toediening van deze stoffen CP en IP werden uitgescheiden over verschillende perioden vanaf het begin van de werkzaamheden Dit kan duiden op verschillende blootstellingsmomenten, verschillende opnameroutes en/of interindividuele verschillen in de biotransformatie en uitscheidingssnelheden van deze stoffen

Alhoewel speciale richtlijnen en beschermende maatregelen zijn geintroduceerd om het ziekenhuispersoneel te beschermen tijdens het omgaan met cytostatica blijkt dat dit de opname van deze stoffen niet voorkomt Vandaar dat in één studie aanvullende beschermende maatregelen zijn genomen om te onderzoeken in hoeverre de ontwikkelde methoden in interventiestudies konden worden gebruikt De maatregelen hadden betrekking op het aanpassen van de laf-kast, het gebruik van speciale maskers, het gebruik van twee paar handschoenen over elkaar en het vervangen van 5FU in ampullen door 5FU in flacons Het effect van deze aanvullende maatregelen is vergeleken met de resultaten uit de voorafgaande studie waarin deze maatregelen nog niet waren ingevoerd De resultaten tonen aan dat de aanvullende beschermende maatregelen de uitwendige blootstelling aan CP hebben verminderd De gemiddelde CP excretiesnelheid voor en na de interventie bedroeg respectievelijk 1,44 en 0,16 μg per dag Het verschil was niet statistisch significant

Tabel 1. Een overzicht van verschillende studies waarbij de uitscheidingssnelheid van CP in de urine van (ziekenhuis)personeel dat werd blootgesteld aan cytostatica is bepaald

groep	aantal personen	periode van urine verzamelen (dagen)	gemiddelde (range) uitscheidingssnelheid[a] CP in urine (µg/dag)	referentie
verpleegkundigen	2	57[b]	0,47 (0,43-0,51)[c]	[1]
ziekenhuispersoneel	20	4	0,39 (0-2,5)	[2]
apothekersassistenten	2	2	0	hoofdstuk 2.1
apothekersassistenten/ verpleegkundigen	18	1-2	0,05 (0-0,5)	hoofdstuk 2.2
proefdierverzorgers	4	2-5	0,05 (0-0,2)	hoofdstuk 2.4
apothekersassistenten[d]	9	1-2	1,44 (0 10)	hoofdstuk 2.5
apothekersassistenten[d]	9	5	0,16 (0-0,51)	hoofdstuk 2.6
verpleegkundigen	8	1	0,79 (0-2,9)	hoofdstuk 3.1
apothekersassistenten	1			
schoonmaaksters	2			
verpleegkundigen	7	2-4	0,80 (0-4,2)	
apotheekpersoneel/ verpleegkundigen	21	1-5	5,2 (0-38)	[3]
apothekersassistenten	8	8-16	0,18 (0,01-0,53)	hoofdstuk 5.1

[a] de uitscheidingssnelheid voor niet detekteerbare hoeveelheden is op 0 gesteld
[b] beide verpleegkundigen hebben urine verzameld over een totale periode van 57 dagen
[c] gemiddelde en range zijn berekend door van een gemiddelde over 28½ dagen uit te gaan
[d] dezelfde personen

[1] Evelo CTA, Bos RP, Peters JGP, Henderson PTh. Urinary cyclophosphamide assay as a method for biological monitoring of occupational exposure to cyclophosphamide. Int Arch Occup Environ Health (1986) 58:151-155.
[2] Hirst M, Tse S, Mills DG, Levin L. Occupational exposure to cyclophosphamide. The Lancet (1984):186-188.
[3] Ensslin AS, Stoll Y, Pethran A, Pfaller A, Rommelt H, Fruhmann G. Biological monitoring of cyclophosphamide and ifosfamide in urine of hospital personnel occupationally exposed to cytotoxic drugs. Occup Environ Med (1994) 51:229-233.

Voor het bepalen van MTX in urine van beroepsmatig blootgestelde personen is een fluorescentie polarisatie immunoassay (FPIA) methode ontwikkeld. FPIA wordt frequent gebruikt voor het monitoren van MTX in serum van patiënten. De FPIA methode is op een zodanige wijze gemodificeerd dat MTX snel en efficiënt in urinemonsters van blootgestelde personen kon worden gemeten. De urinemonsters worden gezuiverd met solid-phase extractie. Concentraties

van minimaal 1 equivalent MTX/ml urine konden worden gedetecteerd
Er werd een GC-MS methode ontwikkeld voor het bepalen van α-fluoro-ß-alanine (FBAL), de hoofdmetaboliet van 5FU, in urine van beroepsmatig blootgestelde personen Hiertoe wordt FBAL gederivatiseerd met S-ethyltrifluorothioacetate en aangezuurd n-butanol De detectiegrens is ongeveer 60 ng/ml urine doch kan nog verder worden verlaagd tot 6 ng/ml door gebruik te maken van GC-MSMS
De ontwikkelde methoden voor biologische monitoring van blootstelling aan 5FU en MTX zijn gevalideerd in een farmaceutisch bedrijf waar werknemers betrokken waren bij de formulering van deze stoffen (afwegen, mengen, filtreren, en afvullen) Vijftig microgram FBAL werd aangetoond in een urinemonster van een medewerker die betrokken was bij het afwegen van 5FU De uitscheidingssnelheid van MTX over 72-96 uur was hoger voor de blootgestelde groep (gem 4,2 µg MTX-equivalenten/24 uur, range 1,5-7,0 µg MTX-equivalenten/24 uur) dan voor de controle groep (gem 3,2 µg MTX-equivalenten/24 uur, range 1,5-6,6 µg MTX-equivalenten/24 uur)

De ontwikkelde methoden zijn in de praktijk geschikt gebleken voor biologische monitoring van beroepsmatige blootstelling aan CP, IP, 5FU and MTX De methoden werden toegepast in verschillende studies Hierbij is gebleken dat de methoden geschikt waren voor het meten van de opname van deze stoffen in het lichaam van de betrokken personen De resultaten van de verschillende studies hebben aangetoond dat gedurende de bereiding en de toediening van cytostatica, waaronder CP, het personeel inwendig werd blootgesteld aan CP Opname van CP werd zelfs gevonden bij apothekersassistenten die cytostatica hebben bereid met uitzondering van CP Een eenduidige blootstellingsroute kon niet worden aangegeven alhoewel er sterke aanwijzingen waren dat inhalatie niet de enigste blootstellingsroute was Vooral de resultaten waarbij CP in de urine van ziekenhuispersoneel werd aangetoond terwijl deze geen CP hadden bereid en/of toegediend, toont de meerwaarde van deze biologische monitoringsmethode aan

Biologische effecten in werknemers blootgesteld aan cytostatica

De uitscheiding van CP in de urine en de aanwezigheid van chromosoomafwijkingen in perifere lymphocyten werden bepaald in 4 groepen ziekenhuispersoneel met verschillende blootstelling Het doel van deze studie was na te gaan of er een mogelijk verband zou bestaan tussen de opname van CP en een vroege biologische respons in de vorm van genetische schade De 4 groepen waren. 17 Nederlandse en 11 Tjechische blootgestelde ziekenhuismedewerkers, die beroepsmatig handelingen met cytostatica uitvoerden, en 35 Nederlandse en 23 Tjechische controle personen werkzaam in dezelfde

ziekenhuizen, doch niet betrokken bij de bereiding en de toediening van cytostatica. De groepen werden onderverdeeld in rokers en niet-rokers.
Binnen de Nederlandse groepen was het percentage afwijkende cellen (3,2 versus 2,0) en het aantal breuken per cel (0,042 versus 0,023) verhoogd voor rokers in vergelijking met niet-rokers. Het percentage afwijkende cellen was hoger in de Nederlandse blootgestelde groep dan in de Nederlandse controle groep (2,6 versus 2,1). Binnen de Tjechische groepen was het percentage afwijkende cellen (2,7 versus 1,8) en het aantal breuken per cel (0,034 versus 0,020) hoger in de blootgestelde groep dan in de controle groep. Dit werd met name veroorzaakt door de Nederlandse en de Tjechische rokers. De resultaten wezen op een additief effect van blootstelling en roken in de Nederlandse groepen en een meer dan additief effect in de Tjechische groepen.
In de urine van 3 van de 11 Nederlandse blootgestelde medewerkers varieerde de uitscheidingssnelheid van CP in de urine van 0,1 tot 0,5 μg/24 uur. Hogere uitscheidingssnelheden werden gevonden bij 8 van de 11 Tjechische medewerkers variërend van 0,1 tot 2,9 μg/24 uur. Er is geen correlatie aangetoond tussen de hoeveelheden CP die met de urine werden uitgescheiden en het percentage afwijkende cellen en het aantal breuken per cel.
De resultaten suggereren dat uitscheidingsnelheden van CP in de urine tot maximaal 2,9 μg/24 uur op de lange termijn zouden kunnen resulteren in biologische effecten zoals chromosoomafwijkingen. Aangezien chromosoomafwijkingen als indicatoren van genetische schade worden beschouwd, tonen deze uitscheidingssnelheden niet alleen opname van CP aan, maar kunnen ze ook wijzen op een ernstig gezondheidsrisico, met name voor rokers.

Om de mogelijke nefrotoxische effecten als gevolg van de beroepsmatige blootstelling aan cytostatica te onderzoeken, werden de vroege effectparameters retinol-bindend eiwit (RBP) en albumine (ALB) bepaald in de urine van 11 ziekenhuismedewerkers die betrokken waren bij de bereiding en toediening van cytostatica. . Een groep van 23 ziekenhuismedewerkers niet betrokken bij de bereiding en toediening van cytostatica diende als controle groep. Er werd geen verschil tussen de blootgestelde en de controle groep aangetoond met betrekking tot RBP (7,9 μg/mol CREAT-U versus 8,8 μg/mol CREAT-U) en ALB (0,44 mg/mol CREAT-U versus 0,30 mg/mol CREAT-U). Alhoewel werd aangetoond dat het ziekenhuispersoneel was blootgesteld aan CP, (excretiesnelheid van CP in de urine varieerde van 0,1-2,9 μg/24 uur), toonden de resultaten van deze studie aan dat deze blootstellingsniveaus geen meetbare nefrotoxische effecten tot gevolg hadden.

Toxicokinetische studies met cyclofosfamide (opname, biotransformatie en excretie)

Inhalatie en huidopname worden verondersteld de belangrijkste opnameroutes van beroepsmatige blootstelling aan cytostatica te zijn. In een studie werden de uitscheidingssnelheden van onveranderd CP in de urine van ratten bepaald na intratracheale toediening en dermale applicatie. Deze blootstellingroutes werden gekozen om de mogelijke beroepsmatige blootstellingsroutes na te bootsen. De resultaten tonen aan dat de maximale uitgescheiden hoeveelheid CP (ongeveer 5% van de dosis werd onveranderd uitgescheiden) onafhankelijk was van de blootstellingsroute. De laagste uitscheidingssnelheid werd gevonden na dermale applicatie.
Deze resultaten en die van Hirst et al. (1984) in dermaal behandelde vrijwilligers, tonen aan dat CP gemakkelijk de huid van ratten en de mens passeert [1]. Om deze reden wordt dermale blootstelling als een mogelijke blootstellings- en opnameroute voor CP beschouwd.

De in vitro biotransformatie van CP werd onderzocht met S-9 leverfracties van humane donoren. Daartoe werd een methode ontwikkeld waarmee de CP metabolieten norstikstof mosterd (NNM), 4-ketocyclofosfamide (KCP) en carboxyfosfamide (CAR) in incubatiemengsels konden worden bepaald. Na vloeistof-vloeistof extractie en derivatisering, werden de metabolieten gemeten met GC-TSD. De resultaten tonen grote interindividuele verschillen in de vorming van NNM en CAR aan. KCP kon niet worden gedetecteerd.

De biotransformatie van CP is ook onderzocht in vitro met S9 rattelever fracties. Het doel was na te gaan of er een mogelijke invloed van de omgevingsfactoren roken en alcoholgebruik op de biotransformatie van CP zou bestaan. De effecten zijn nagebootst door ratten te behandelen met respectievelijk ß-nafthoflavon en ethanol. De invloed van geslacht en suppletie met NAD en GSH werd eveneens onderzocht. De resultaten tonen aan dat de in vitro biotransformatie van CP in de rat sterk wordt beïnvloed door het geslacht en de suppletie met NAD en GSH. Voorbehandeling met ß-nafthoflavon en ethanol beïnvloedde de biotransformatie niet. De resultaten suggereren dat de omgevingsfactoren roken en alcoholgebruik een verwaarloosbare invloed hebben op de biotransformatie van CP in de mens.

Risicoschatting van beroepsmatige blootstelling aan cyclofosfamide

Er werd een kankerrisicoschatting als gevolg van beroepsmatige blootstelling aan het genotoxisch carcinogene cytostaticum CP uitgevoerd waarbij 2 benaderingen werden gevolgd. In het ene geval werd uitgegaan van gegevens van een carcinogeniteitsstudie bij proefdieren. In het andere geval werd uitgegaan van gegevens over de ontwikkeling van primaire en secundaire tumoren bij patiënten behandeld met CP. In beide studies werden met name blaastumoren en leukemiën gevonden. De opname van CP in de beroepssituatie werd geschat op basis van de uitscheiding van CP in de urine van verschillende groepen werknemers die blootgesteld waren aan cytostatica waaronder CP. De opname varieerde van 3,6-18 μg per dag. Het kankerrisico werd bepaald op basis van de dosis-respons gegevens van de proefdierstudie en de patiëntenstudies. Hierbij zijn verschillende aannames gedaan. Het kankerrisico gebaseerd op de proefdierstudie is berekend voor een persoon van 70 kg met een blootstellingsperiode van 40 jaar, 200 dagen per jaar. Het "lifetime" risico op blaaskanker in mannen en leukemiën in mannen en vrouwen was vergelijkbaar en varieert van 100-600 per miljoen bij een dagelijkse dosis van 3,6-18 μg. Het kankerrisico gebaseerd op de patiëntengegevens werd berekend door de 10-jaars cumulatieve incidentie per g CP te vermenigvuldigen met de gemiddelde opname over 10 jaar, 200 dagen per jaar varierend van 7,2 tot 36 mg. Op basis van secundaire tumordata is het risico op leukemiën in vrouwen berekend op 17-100 per miljoen over 10 jaar. Een marginaal lager risico voor blaaskanker en leukemiën in mannen en vrouwen van 15-76 werd gevonden wanneer primaire tumor data werden gebruikt. Omgerekend betekent dit dat het kankerrisico verkregen op basis van zowel de proefdiergegevens als de patiëntengegevens overeenkomt met ongeveer 1,4 tot 10 per miljoen per jaar.

In Nederland wordt voorgesteld bij beroepsmatige blootstelling aan kankerverwekkende stoffen te streven naar een kankerrisico per stof van 1 extra kankergeval per miljoen per jaar (streefrisiconiveau) en geen hoger risico dan 100 per miljoen per jaar (verbodsrisiconiveau). Uit de risicoschatting op basis van de proefdiergegevens en de patiëntengegevens blijkt dat het streefrisiconiveau wordt overschreden, maar dat het risico nog onder het verbodsrisiconiveau blijft. Wel dient te worden opgemerkt dat in enkele onderzochte groepen hogere uitscheidingssnelheden voor CP zijn gevonden dan de gemiddelde CP uitscheidingssnelheid van 0,18 μg waarop deze risicoschatting is gebaseerd (Tabel 1). Een voorbeeld hiervan is een studie met 7 verpleegkundigen waarbij een ongeveer 5 keer zo hoge gemiddelde excretiesnelheid van 0.80 μg/dag werd gevonden. Dit betekent een overeenkomstig 5 keer hoger kankerrisico. Van belang is nogmaals op te

merken dat deze risicoschatting uitsluitend betrekking heeft op kanker.
Ondanks de introductie van streef- en verbodsrisiconiveaus dient te worden benadrukt dat het de taak van werkgevers, werknemers en de overheid is ervoor te zorgen dat elke vorm van beroepskanker als gevolg van blootstelling aan cytostatica (en andere kankerverwekkende stoffen) moet worden voorkomen.

Tot slot

De methoden die in het kader van het hier beschreven onderzoek zijn ontwikkeld en gevalideerd, kunnen een belangrijke bijdrage leveren aan het opsporen van beroepsmatige blootstelling aan cytostatica en daarmee aan het reduceren van de mogelijk hiermee gepaard gaande risico's voor de gezondheid van betrokkenen.

Curriculum vitae

Paul Sessink werd op 10 december 1960 geboren in Ulft (gemeente Gendringen) Het middelbaar onderwijs volgde hij aan het Isala College in Silvolde In 1975 verhuisde hij naar Opglabbeek in België De middelbare schooltijd werd voortgezet aan het Stedelijk Lyceum in Maastricht en aan het St Jan Berchmanscollege, een jongensschool, in Genk (België) In 1978 verhuisde hij terug naar Ulft en vervolgde de middelbare school aan het Isala College In 1980 behaalde hij het diploma Atheneum en begon hij met de studie Scheikunde aan de Katholieke Universiteit Nijmegen (KUN) Het kandidaatsdiploma Scheikunde werd behaald in 1984 en het doctoraaldiploma Scheikunde in 1988 De doctoraalfase omvatte 2 hoofdrichtingen organische chemie en toxicologie De stage organische chemie werd doorlopen bij de vakgroep Organische Chemie II van de KUN (prof dr B Zwanenburg) Tijdens deze stage deed hij onderzoek aan de Favorskii ester als synthon voor de synthese van optisch actieve cyclopentenonen De stage toxicologie werd doorlopen bij de vakgroep Toxicologie aan de KUN (prof dr P Th Henderson) Tijdens deze stage onderzocht hij de mogelijkheden van histidine methylering van serum albumine als dosismonitor voor expositie aan methylerende agentia Van 1 juni 1988 t/m 15 december 1993 werkte hij bij de vakgroep Toxicologie (KUN) aan het promotieonderzoek waarvan in dit proefschrift verslag wordt gedaan In dezelfde periode volgde hij een aantal cursussen van de Post-doctorale Opleiding Toxicologie en verwierf hij de status van artikel 9 functionaris van de Wet op de Dierproeven In 1995 ontving hij een prijs voor zijn wetenschappelijke werk met betrekking tot monitoring van beroepsmatige blootstelling aan cytostatica tijdens ISOPP IV (International Symposium on Oncology Pharmacy Practice) in Hamburg

Ook in maatschappelijk opzicht was en is hij actief In 1979 richtte hij samen met een aantal anderen de Oudheidkundige Vereniging Gemeente Gendringen op Vanaf de oprichting tot 1987 was hij secretaris van de vereniging Omstreeks dezelfde periode werd hij lid van D66 en richtte samen met anderen de plaatselijke afdeling van D66 in de gemeente Gendringen op Van 1986-1990 was hij regionaal wedstrijdleider en van 1989-1995 landelijk wedstrijdleider van de Algemene Nederlandse Sjoelbond Van 1987-1994 was hij penningmeester van Stichting Scouting Anne Frank uit Nijmegen In 1994 werd hij gekozen als raadslid voor D66 in de gemeente Wijchen In die hoedanigheid is hij vanaf 1995 tevens algemeen bestuurslid van het Knooppunt Arnhem Nijmegen

In 1994-1995 nam hij deel aan de eerste cursus "Kennis en Ondernemerschap" van de vakgroep Bedrijfswetenschappen en het Transferbureau van de KUN. Het door hem tijdens deze cursus opgestelde bedrijfsplan werd met de 1e prijs beloond. Op basis van het bedrijfsplan begon hij op 1 april 1995 het adviesbureau "Exposure Control", dat adviseert omtrent de gevaren van blootstelling aan giftige stoffen in het algemeen en aan cytostatica in het bijzonder. De in dit proefschrift beschreven methoden van omgevings- en biologische monitoring van cytostatica worden hierbij toegepast om de blootstelling aan deze stoffen in kaart te brengen. Vanaf 1 april 1995 is hij tevens directeur van de BVOR (Belangenvereniging voor Verwerkingsbedrijven van Organische Reststoffen), een vereniging van bedrijven die zich bezighoudt met het verwerken (composteren) van groenafval.

Hij is getrouwd met Ingrid Ruesink. Hun zoon heet Tom.

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Exposure Control

is een adviesbureau gespecialiseerd op het gebied van monitoring van beroepsmatige blootstelling aan cytostatica

Exposure Control concentreert haar diensten op:
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- het opstellen en uitvoeren van meetprogramma's (omgevings- en biologische monitoring)
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Voor nadere informatie kunt u contact opnemen met:

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Stellingen behorende bij het proefschrift:

Monitoring of Occupational Exposure to Antineoplastic Agents

1. De beschreven methoden voor omgevings- en biologische monitoring van cyclofosfamide, ifosfamide, 5-fluorouracil en methotrexaat zijn bij de huidige expositieniveau's bruikbaar voor het opsporen van contaminatie van de werkomgeving en voor het vaststellen van de opname van deze cytostatica in het lichaam van personen die beroepsmatig met deze stoffen in aanraking kunnen komen.
   Dit proefschrift.

2. Beroepsmatige blootstelling aan cyclofosfamide kan het beste worden vastgesteld met behulp van biologische monitoring.
   Dit proefschrift.

3. Ondanks de uitgebreide arbeidshygiënische maatregelen en voorzieningen die in Nederland momenteel worden toegepast, worden apothekersassistenten en oncologieverpleegkundigen blootgesteld aan cytostatica.
   Dit proefschrift.

4. De relatief grote hoeveelheden cyclofosfamide in de urine in de studie van Ensslin et al. in vergelijking met de resultaten in dit proefschrift en de vele, in verband met matrixpoblemen niet geanalyseerde monsters, doet vermoeden dat er sprake is geweest van een identificatie probleem. Deze problemen zouden kunnen worden voorkomen door gebruik te maken van GC-MS(MS).
   Ensslin et al., Occupational and Environmental Medicine 1994;51:229-233.

5. De Europese gedachte is nog ver te zoeken als het gaat om uniformiteit in het opstellen van een richtlijn met betrekking tot het beroepsmatig omgaan van ziekenhuismedewerkers met cytostatica.

6. Gezien het ontbreken van duidelijke gegevens met betrekking tot gezondheidsrisico's bij zwangeren als gevolg van blootstelling aan cytostatica verdient het aanbeveling apotheekmedewerkers en oncologieverpleegkundigen met een kinderwens vrij te stellen van handelingen met cytostatica.

7. Voor de genotoxisch carcinogene stof cyclofosfamide dient het verbods- en streef-risiconivo van een extra kans op kanker van respectievelijk 4 per 1000 en 4 per 10.000 personen per jaar te worden vastgesteld op basis van de uitscheiding van cyclofosfamide in de urine en niet op basis van concentraties cyclofosfamide in de omgevingslucht.
Berekening van het risico op kanker door beroepsmatige blootstelling aan genotoxisch carcinogene stoffen. Advies van de Commissie WGD van de Gezondheidsraad (1995/06WGD).

8. Alhoewel het ontwikkelen van methoden voor biologische-effect monitoring ongetwijfeld wetenschappelijk interessant is, is het de vraag in welke mate een aantal hiervan in de arbozorg zullen/kunnen worden toegepast met name vanwege de moeilijke interpretatie van de data en de hoge kosten van deze methoden. Dit in tegenstelling tot methoden van biologische monitoring.

9. Het onderwerken van bermmaaisel in de landbouw is uit milieuoogpunt ongewenst omdat dit gepaard gaat met het gebruik van meer bestrijdingsmiddelen.

10. Een hogere opkomst bij verkiezingen kan worden gerealiseerd door burgers voor het uitbrengen van hun stem met air miles te "belonen".

Paul Sessink.

Nijmegen, 4 november 1996.