Clinical Pharmacology
of
Ifosfamide and Metabolites

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Klinische Farmacologie van Ifosfamide en Metabolieten
(met een samenvatting in het Nederlands)

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Preface

Despite significant advances in the treatment of various types of cancer over the past decades, there is still a fundamental need to improve tumour response rates and survival in patients with advanced cancer. Besides development of new chemotherapeutic agents, existing therapies could be modified to achieve these goals. The intensity of cancer treatment is of paramount importance for the response of the patient. Nowadays, in most therapies systemic exposure is solely calculated on the basis of drug-dose administered per unit time (dose-intensity). This does not take into account pharmacokinetic variability. Anticancer drugs generally display significant pharmacokinetic variability and systemic exposure can differ several-fold amongst patients given an equivalent dosage possibly resulting in differences in success of treatment.[1]

The anticancer agent ifosfamide is a prodrug that requires metabolic activation to exert its cytotoxic activity. The first step in the bioactivation pathway occurs mainly in the liver by action of the cytochrome P450 producing 4-hydroxyifosfamide. This intermediate is subject to further activation to yield the alkylating metabolite ifosforamide mustard with concomitant release of the urotoxic metabolite acrolein. Ifosfamide is also deactivated to 2- and 3-dechloroethylifosfamide, which is accompanied by an equimolar formation of the neurotoxic chloroacetaldehyde.[2] Ifosfamide pharmacokinetics is further characterized by a time-dependent increase in metabolic clearance explained by a mechanism of autoinduction of the hepatic oxygenase system.

Pharmacokinetics of ifosfamide and metabolites show a large inter- and intraindividual variability. The determinants (patient characteristics, concomitant medication, autoinduction, infusion schedule, dose, etc.) of this variability were not yet known in detail. Furthermore, relationships between pharmacokinetics and pharmacodynamics were scanty. Particularly correlations between plasma levels of metabolites and toxicity and/or antitumour activity were lacking. On the one hand this may be due to the lack of tools for the investigation the determinants of varibility and on the other hand the lack of relatively complex methodologies necessary for the analysis of metabolites in biological matrices. The analysis is also hampered by the limited stability of the analytes in plasma and urine. Therefore, new sensitive and validated assay, which can be routinely used, needed to be developed for the quantitative determination of ifosfamide, 2- and 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard in biological matrices. These methods were based on the techniques of gas chromatography, either with nitrogen-phosphorous detection or ion-trap mass spectrometry and liquid chromatography (after derivatization).

The implementation of pharmacokinetic monitoring of ifosfamide and metabolites in clinical studies offers a unique possibility to study pharmacokinetic variability and pharmacokinetic-pharmacodynamic relationships in ifosfamide therapy. However, for practical and ethical reasons extensive pharmacokinetic and pharmacodynamic studies in a large number of often critically ill patients are not possible. Therefore, there was a need for an approach with the help of which description of the relevant pharmacokinetic and pharmacodynamic relationships and of their variability is possible on the basis of sparse data (few data points-per patient) collected under unbalanced designs. A population approach also allows therapeutic drug monitoring of ifosfamide. Therapeutic drug monitoring can help in further tailoring ifosfamide

treatment to the specific needs of the individual patient.
The aims of the research described in this thesis were to develop bioanalytical methods for determination of ifosfamide and its metabolites, to build population pharmacokinetic models for these compounds and to apply the techniques in various clinical studies on ifosfamide. New insights in the pharmacokinetics of ifosfamide and its metabolites may contribute to the development of new ifosfamide therapies with increased efficacy and/or decreased toxicities.

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Chapter 1.1

Clinical pharmacokinetics and pharmacodynamics of

ifosfamide and its metabolites

Summary

This review will discuss several issues in the clinical pharmacology of the anti-tumour agent ifosfamide and its metabolites. Ifosfamide is effective in a large number of malignant diseases. Its use, however, can be accompanied by haematological, neuro- and nephrotoxicities. Since its development in the middle of the 1960's, most of its extensive metabolism has been elucidated. Identification of specific isoenzymes responsible for ifosfamide metabolism may lead to an improved efficacy/toxicity ratio by modulation of the metabolic pathways. Whether ifosfamide is specifically transported by erythrocytes and which activated ifosfamide metabolites play a key role in this transport is currently being debated. In most clinical pharmacokinetic studies the phenomenon of autoinduction has been observed, but the mechanism is currently not completely understood. Assessment of the pharmacokinetics of ifosfamide and metabolites has long been impaired by the lack of reliable bioanalytical assays. The recent development of improved bioanalytical assays has changed this dramatically allowing extensive pharmacokinetic assessment, identifying key issues like population differences in pharmacokinetic parameters, differences in elimination dependent upon route and schedule of administration, implications of the drug chirality and interpatient pharmacokinetic variability. The mechanisms of action of cytotoxicity, neurotoxicity, urotoxicity and nephrotoxicity have been pivotal issues in the assessment of the pharmacodynamics of ifosfamide. Correlations between the new insights into ifosfamide metabolism, pharmacokinetics and pharmacodynamics rationalize the further development of therapeutic drug monitoring and dose individualization of ifosfamide treatment.

Development

The oxazaphosphorines ifosfamide, *N*,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxaza-

phosphorin-2-amine 2-oxide (Holoxan®), cyclophosphamide and to a lesser extent, trofosfamide, mafosfamide, sufosfamide and glufosfamide play an important role in the treatment of various tumours.[1-3] Oxazaphosphorines are nitrogen mustard derivatives, as depicted in figure 1. During World War I chemical warfare with mustard gasses had produced leukocytopenia, bone marrow aplasia and lymphatic tissue dissolution. Gilman, Goodman and Dougherty evaluated these effects of nitrogen mustard on transplanted lymphosarcoma in murine models during the early 1940's.[4,5] Shortly after, the first modern clinical testing of chemotherapy led to the discovery of cyclophosphamide. Ifosfamide has been developed in the middle of the 1960's as structural isomer of cyclophosphamide and introduced as an anti-cancer agent in the early 1970's.[6,7]

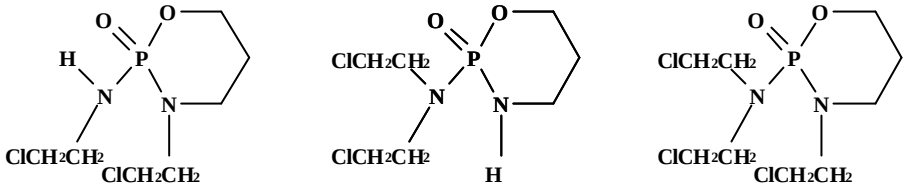

Figure 1. *Oxazaphosphorines*

Early pre-clinical testing of ifosfamide proved superior activity over cyclophosphamide, but initial trials in the 1970's resulted in severe dose-limiting haemorrhagic cystitis.[8] Although urotoxicity could be minimized by fractionated dosage regimens and increased hydration, it remained the main dose-limiting toxicity. In the 1980's it became clear that the ifosfamide metabolite acrolein was responsible for the urotoxicity.[2] The antidotes sodium 2-mercaptoethane sulphonate (mesna, Uromitexan®) and acetylcysteine were developed, which specifically react with acrolein in the urinary tract thereby preventing haemorrhagic cystitis without hampering ifosfamide cytotoxicity in blood and tissue.[9] Co-administration of mesna enabled further (high dose) regimen development with ifosfamide in clinical trials.[10]

Metabolism

Ifosfamide is a prodrug which needs activation via the cytochrome P450 enzymatic system in the liver to 4-hydroxyifosfamide. 4-Hydroxyifosfamide exists in equilibrium with its tautomeric form aldoifosfamide. Aldoifosfamide can be converted into the final alkylating agent ifosforamide mustard with concurrent acrolein formation, as depicted in figure 2. After ß-elimination of acrolein from 4-hydroxyifosfamide, the cytotoxic ifosforamide mustard is formed.[11] Acrolein is held responsible for the urotoxic effects of ifosfamide. Alkylation of DNA by ifosforamide mustard can cause cross-links, resulting in cytotoxicity. Ifosforamide mustard is also known to yield the metabolites chloroethylamine and 1,3-oxazolidine-2-one.[12]

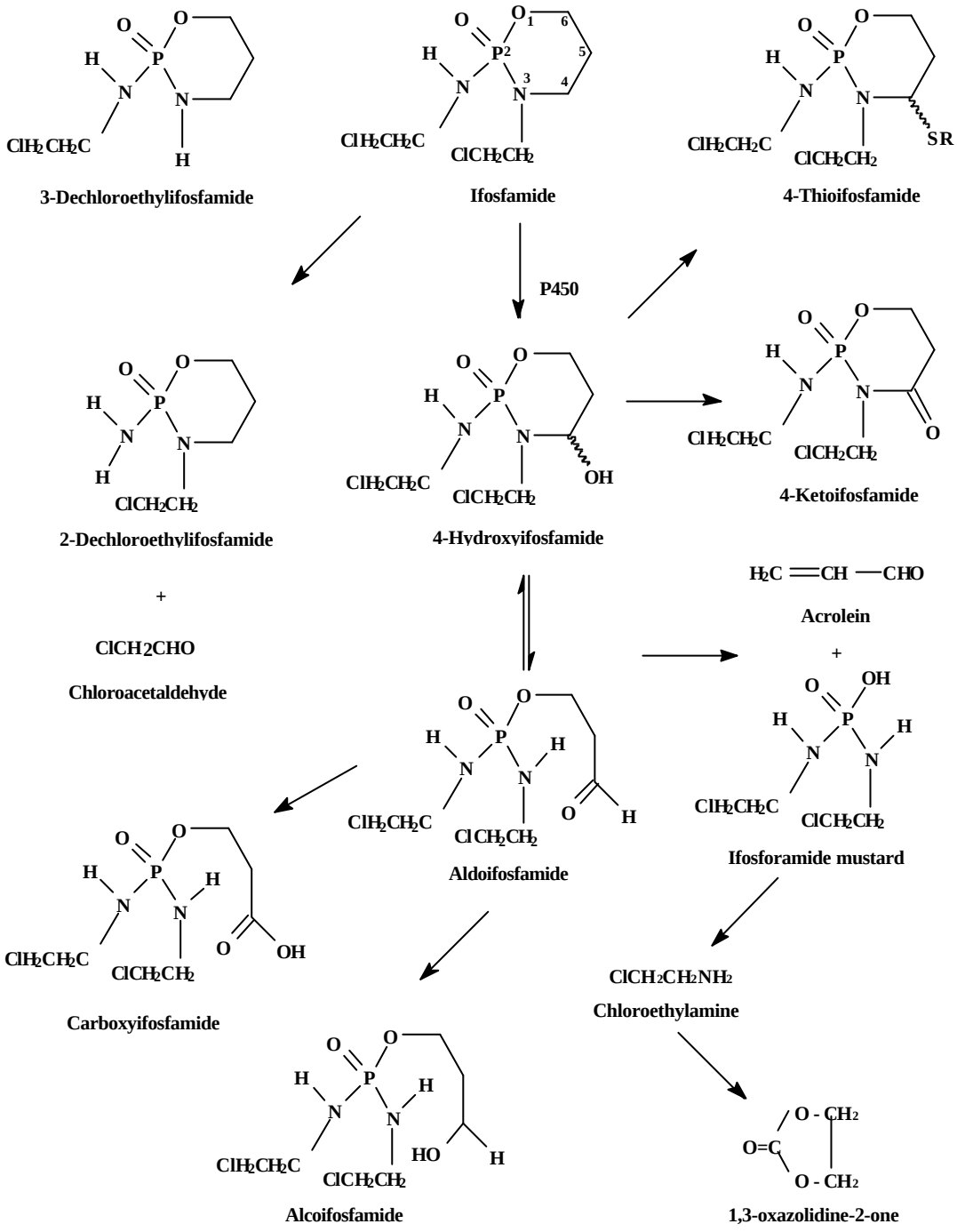

Figure 2. *Ifosfamide metabolism*

Furthermore, 4-hydroxyifosfamide is metabolized to the inactive metabolites 4-ketoifosfamide and 4-thioifosfamide, and its tautomer aldoifosfamide to carboxy-ifosfamide and alcoifosfamide. Ifosfamide is also subject to deactivation routes that involve removal of a chloroethylgroup from either the exo- or endocyclic nitrogen atom to form non-toxic 2-dechloroethylifosfamide and 3-dechloroethylifosfamide, respectively. Dechloro-ethylation is achieved by a side-chain oxidation, releasing an equimolar amount of the potentially neurotoxic chloroacetaldehyde[13], as represented in figure 2. Chloroacetaldehyde may also

be involved in the urotoxicty observed in ifosfamide treatment.[14]

Cytochrome P450

Much research has been devoted to the metabolism of ifosfamide in the rat. Activation and deactivation of ifosfamide *in vivo* and in rat liver microsomes proved to be mediated by different cytochrome P450 isoenzymes 3A (CYP3A) plus CYP2B1/CYP2C11 and CYP3A, respectively.[15-18] Proof of differential enantioselective metabolism in rats was given, the specific isoenzymes responsible for metabolism in rats, however, differed significantly from humans.[19] Furthermore, corresponding P450 enzymes may perform different metabolic functions in different species, which hampers extrapolation of results obtained from laboratory animals to humans. In humans both activation (hydroxylation) and deactivation (dechloroethylation) demonstrated to be mediated by the CYP3A4.[20-22] Paradoxically, the mixed function CYP3A4 performs both desired hydroxylation and undesired dechloroethylation, making it a target for development of specific inhibition or hetero-induction regimens and thereby directing ifosfamide metabolism to a more favourable toxicity-efficacy profile. Other isoenzymes have also been implicated with ifosfamide metabolism. Human liver microsomes have demonstrated enantioselective metabolism.[23] This *in vitro* study supported the conclusion that CYP2B6 and not CYP3A4 were responsible for the formation of (S)-3- and (S)-2-dechloroethylifosfamide. Another study suggested that (R)-ifosfamide might have a more favourable efficacy/toxicity profile, due to enantioselective differences of CYP3A4 and CYP2B6.[24] The activity of CYP2B6 in the *in vitro* metabolization of ifosfamide was also demonstrated in isolated cDNAs.[25] Furthermore, CYP2B1 has also proven to metabolize ifosfamide, yielding a cytotoxic effect in human cell lines *in vitro.*[26] There is no definite answer, whether CYP3A4 is the only *in vivo* relevant enzyme in the metabolism of ifosfamide, or if other isoenzyme contribute. Secondary isoenzyme metabolism may also be important, because a blockade of the main metabolic isoenzyme of ifosfamide by for instance co-administrated drugs could render lower affinity isoenzymes into relevant metabolic pathways. Modulation of metabolism of ifosfamide, however, has not been successful till now.[27] Failure of modulation could be explained by the simultaneous administration of phenobarbital with ifosfamide, which did not allow for enzyme induction to develop.

Another way to direct metabolism of ifosfamide is gene transfer of CYP in tumour cells.[26,28] Especially, the tumour bystander effect has been identified as a possible effective strategy. The tumour bystander effect will result in more widespread cytotoxicity in the tumour. Although only a small subset of tumour cells will be successfully transfected, their CYP upregulation will provide local elevated metabolism of ifosfamide, thus spreading the diffusable cytotoxic metabolites to adjacent and more distant non-transfected tumour cells and greatly enhancing the anti-tumour effect.[29]

The different isoenzymes that have been identified demonstrate the need to consider interaction with co-administered drugs that are also CYP3A4 substrates. Concomitant therapy with inducers of CYP3A4 (e.g. rifampicin, carbamazepine, phenobarbitone and phenytoin) enhances the expression of this enzyme and may increase metabolic activation, possibly resulting in enhanced efficacy and toxicity.[30] Phenytoin induced a shift in enantioselective dechloroethylation in children, possibly increasing ifosfamide efficacy.[31] In

contrast inhibitors and substrates of CYP3A4 (e.g. erythromycin, ketoconazole, itraconazole, diltiazem, verapamil and cyclosporin) could interfere with the activation of ifosfamide.[32] Vice-versa, ifosfamide could also be able to influence other co-administered drugs, possibly reducing the therapeutic effect of the latter.[33] However, the clinical significance of the changes in pharmacokinetics of or caused by ifosfamide resulting from CYP3A4 inhibition and induction are not well established yet.

Autoinduction

Metabolism of ifosfamide is an autoinducible enzymatic process, resulting in an increased clearance over time.[34] The exact physiological mechanism of the autoinduction of ifosfamide is unknown, but several mechanisms of drug induced metabolic changes have been reported for other compounds. For most of these drugs the amount of enzymes performing the metabolic breakdown is increased via *de novo* synthesis. For instance, the barbiturate pentobarbital induces nortriptyline metabolism by increasing translation of the DNA coding for the enzyme and thereby increasing clearance of systemic nortriptyline. *De novo* synthesis of CYP by pentobarbital was observed after two days.[35] Rapid autoinduction of carbamazepine due to *de novo* synthesis was already demonstrated after 12-24 hours with a maximum after six weeks.[36] Autoinduction of ifosfamide has been observed within 24 hours after start of treatment.[37] However, it is unlikely that an alkylating agent like ifosfamide (which reduces DNA/RNA and protein synthesis) would increase enzyme protein synthesis of CYP. On the other hand, non-transcriptional induction mechanism has also been demonstrated to produce drug induced metabolic changes. Troleandomycin decreased the rate of the CYP3A4 protein degradation by interacting with specific catalytic enzymes, which resulted in an increased pool of CYP3A4 protein.[38] An uncommon interaction of a drug with its metabolising enzyme has been demonstrated for phenobarbital, as it achieved a direct influence on the enzyme.[39] By binding of the drug to a non-catalytic site of the enzyme, the catalytic ability increased due to an altered spin state of the haemoprotein of the CYP. Ifosfamide has been shown to reduce protein synthesis[40] and as such may reduce synthesis of proteins responsible for CYP3A4 degradation/inactivation, which resulted in decreased ifosfamide clearance. The protein reducing ability of ifosfamide and the high rate of autoinduction suggest that this mechanism of autoinduction is the most viable option, but further research is warranted to prove this hypothesis.

Metabolite distribution

In general, pharmacokinetics are mainly assessed using plasma, urine and/or erythrocytes. Erythrocytes are of special interest in the pharmacokinetics of ifosfamide, because they have been proposed to act as transporters of activated ifosfamide and cyclophosphamide.[41-44] Plasma concentrations of 4-hydroxycyclophosphamide, the activated metabolite of cyclophosphamide, could be detected in low but persistent levels. This led to the theory that, 4-hydroxycyclophosphamide is trapped intracellularly and in this way act as the transport form of activated cyclophosphamide.[45,46] This hypothesis was confirmed by comparing hypothetical total cell-kill percentages of 4-hydroxy-cyclophosphamide and phosphoramide

mustard, based upon plasma levels in mice, rats and humans and anti-tumour sensitivity of mice and human cell cultures to these compounds.[11] Extracellular 4-hydroxy-cyclophosphamide exhibits a more profound cytotoxicity than phosphoramide mustard, caused by its higher penetration capacity of the tumour cell membranes. In contrast to these findings other groups have argued that the ifosforamide mustard is the major transport form of ifosfamide, since the anti-tumour activity and selectivity of extracellular delivered ifosforamide mustard was comparable to 4-hydroxyifosfamide.[47] It has also been suggested that ifosfamide is bioactivated in the liver to ifosforamide mustard. Subsequently, ifosforamide mustard is loaded onto or into the erythrocyte.[43] The erythrocyte will transport ifosforamide mustard to the target tissue by interacting with the capillary endothelium and consequently releasing ifosforamide mustard into the target tissue. Furthermore, it was demonstrated that erythrocytes from five ifosfamide treated patients contained as much as 77% of the total whole blood concentration of ifosforamide mustard, resulting in a ratio of the area under the curve of erythrocytes over plasma of 2.7.[43] At present, the implications whether 4-hydroxy-ifosfamide or ifosforamide mustard is the activated transport form of ifosfamide are not clear.

Bioanalysis

Pharmacokinetic assessment of ifosfamide and its metabolites have long been hampered by the lack of reliable, specific and selective bioanalytical methods.[48,49] During the last decade validated assays have been developed enabling quantitative determination of ifosfamide and its metabolites using various techniques, as represented in table 1.[13,50-80] Modern, bioanalytical methods require high sample-throughput, accuracy and precision in a clinically relevant concentration range, when applied in clinical pharmacokinetic studies. Furthermore, activated oxazaphosphorine metabolites have relati-vely short elimination half-lives, which necessitates stabilization steps to prevent undesired (auto-catalytic) degradation.

Table 1. *Methods for the analysis of ifosfamide and its metabolites in various biological matrices, applicable for clinical pharmacokinetic studies.*

Method	Analyte	Matrix	Derivatization	Clean-up	LLQ	Reference
TLC-PD	IF	U	-	SPE	1 µg/ml	Lind et al.[50]
TLC-VIS	IF	U	PBH	-		Norpoth et al.[13]
TLC-PD	IF, DCE, CI, KI, 4OH, IFM	U, C, P	NBP	SPE	1 µg/ml	Boddy et al.[51]
^{31}P-NMR	IF	*in vivo*	-	-		Payne et al.[52]
^{31}P-NMR	IF, 2DCE, 3DCE, CI, IFM	U	-	SPE		Gillard et al.[53]
^{31}P-NMR	IF, 2DCE, 3DCE, KI, 4OH	U	-	-		Martino et al.[54]
HPLC-UV	IF	P	-	SPE	1 µg/ml	Burton et al.[55]
HPLC-UV	IF	P	-	LLE	0.1 µg/ml	Margison et al.[56]
HPLC-UV	IF	P	-	SPE	6 µg/ml	Zolezzi et al.[57]
HPLC-UV	2DCE, 3DCE	U	-	LLE	2-5 µM	Goren et al.[58]
HPLC-UV	r/s-IF	P	-	SPE	2.50 µg/ml	Corlett et al.[59]
HPLC-UV	r/s-IF	P	-	LLE	10 µg/ml	Masurel et al.[60]
HPLC-UV	CA	P	TU	SPE	1 nM	Kaijser et al.[61]
HPLC-UV	4OHIF	P	3AP	-	50 nM	Kaijser et al.[62]
HPLC-UV	4OHIF	WB	3AP	-	0.1 µM	Kurowski et al.[63]
HPLC-UV	4OHIF	P, E	SCZ	LLE	0.1 µg/ml	Kerbusch et al.[64]

HPLC-UV	IFM	P	DDTC	SPE	0.45 µM	Kaijser et al.[65]
HPLC-UV	IFM, IF	P	DDTC	LLE	0.1 µg/ml	Kerbusch et al.[66]
GC-NPD	IF	P	-	LLE	0.5 µg/ml	Mehta et al.[67]
GC-NPD	IF	P	-	LLE	1 µg/ml	Talha et al.[68]
GC-NPD	IF, 2DCE, 3DCE	P, U	-	LLE	0.05 µg/ml	Kaijser et al.[69]
GC-NPD	IF, 2DCE, 3DCE	P	-	LLE	0.5, 5, 5 µM	Kurowski et al.[63]
GC-NPD	IF, 2DCE, 3DCE	P	-	LLE	0.05 µg/ml	Kerbusch et al.[70]
GC-NPD	r/s-IF, r/s-2DCE, r/s-3DCE	P, U, C	-	LLE	0.02, 0.1, 0.05 µg/ml	Kaijser et al.[71]
GC-NPD	IF, 2DCE, 3DCE, 4OHIF	P	PFBHA	LLE	0.8-3.25 µg/ml	Gourmel et al.[72]
GC-NPD	IFM	P	TFAA	SPE	0.25 µg/ml	Bryant et al.[73]
GC-FID	IF	P	-	LLE	1 µg/ml	Allen et al.[74]
GC-ECD	CA	WB	-	LLE	0.2 µM	Kurowski et al.[63]
FAB-MS	IF-mesna	U	-	LLE		Manz et al.[75]
GC-MS	IF	P	-	LLE	0.01 µg/ml	Lambrechts et al.[76]
GC-MS	IF, 2DCE, 3DCE, KI, CI, IFM, CEA, OXAZ	P	TFAA	SPE LLE	3 ng/ml	Momerency et al.[77]
GC-MS	r/s-IF	P	-	SPE	0.25 µg/ml	Young et al.[78]
GC-MS	r/s-IF, r/s-2DCE, r/s-3DCE	P, U	-	LLE	0.25, 0.5, 0.5 µg/ml	Granville et al.[79]
GC-MS	IF, 2DCE, 3DCE, 4OH, IFM	P	CH, MSTFA, BSTFA, TMSI	LLE SPE	0.1-0.5 µg/ml	Wang et al.[80]

TLC=thin layer chromatography, PD=photography-densitometry, VIS=visible light spectroscopy, ^{31}P-NMR=^{31}P nuclear magnetic resonance spectroscopy, HPLC=high-performance liquid chromatography, UV=ultraviolet detection, GC=gas chromatography, NPD=nitrogen phosphorus selective detection, FID=flame ionization detection, ECD=electron capture detection, FAB=fast atom bombardment, MS=mass spectrometry, IF=ifosfamide, 2DCE=2-dechloroethylIF, 3DCE=3-dechloroethylIF, CI=carboxyIF, KI=ketoIF, 4OH=4-hydroxyIF, IFM=ifosforamide mustard, CEA=chloroethylamine, OXAZ=1,3-oxazolidine-2-one, P=plasma, U=urine, E=erythrocytes, C=cerebro-spinal fluid, WB=whole blood, PBH=4-pyridinealdehyde-2-benzothiazolylhydrazone, NPB=4-(4-nitro-benzyl)pyridine, TU=thiourea, 3AP=3-aminophenol, SCZ=semicarbazide, DDTC=diethyldithiocarbamate, TFAA=trifluoroacetic anhydride, PFBHA=O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine, CH=cyanohydrin, MSTFA=N-methyl-N-trimethylsilyltrifluoro-acetamide, BSTFA=N,O-bis(trimethylsilyl)trifluoroacetamide, TMSI=N-trimethylsilylimidazole, LLE=liquid-liquid extraction, SPE=solid-phase extraction.

Nowadays several types of analytical separation are used. Thin layer chromatography with various detection methods is easy to apply, but is hampered by cross-selectivity and low sensitivity. A more elaborate and expensive method is nuclear magnetic resonance spectroscopy (NMR), which has been used to identify new metabolites of oxazaphosphorines and determination of ifosfamide concentrations *in vivo.*[52]

More established techniques are high-performance liquid chromatography and gas chromatography. High-performance liquid chromatography is easy to use, but due to its undiscriminating detection at lower wavelengths (200-300 nm) a labour-intensive sample preparation is required to separate the analytes from interfering plasma components. The problems of the rapid auto-catalytic degradation of ifosforamide mustard and 4-hydroxy-ifosfamide and their lack of a suitable chromophore was solved by immediate controlled derivatization.[66,64] Gas chromatography, on the other hand, quantifies volatile analytes. Gas chromatography can be very selective and sensitive, but the analytes need to be thermally stable, excluding 4-hydroxyifosfamide and ifosforamide mustard.[81] Due to its selectivity, for gas chromatography with nitrogen-phosphorus detection a simple liquid-liquid extraction is sufficient.[82] Possible chiral differences in ifosfamide metabolism are suspected, warranting

determination of the enantiomers of ifosfamide and its 2- and 3-dechloroethylifosfamide metabolites.[71,79] During the last decade, the use of mass spectrometric detection (MS) has increased due to its theoretical increased selectivity.[77] A major drawback of these hyphenated techniques is their use of non-commercially available derivatization agents and elaborate sample pre-treatment procedures. In summary, gas chromatography with nitrogen-phosphorus detection is the method of preference for ifosfamide, 2- and 3-dechloroethyl-ifosfamide and high-performance liquid chromatography with ultra-violet detection for the derivatized products of 4-hydroxyifosfamide and ifosforamide mustard.[64,66,70]

Pharmacokinetics

Despite large patient variability in the metabolism of ifosfamide, a general pattern can be observed. Dechloroethylation can account for 25 to 60% of the metabolism of ifosfamide.[11,83] This in contrast to cyclophosphamide, which has only minor oxidative side-chain metabolism. Since both dechloroethylation and hydroxylation are competing metabolic pathways, plasma concentrations of activated metabolites will be lower than with cyclophosphamide. Following intravenous administration, the volume of distribution of ifosfamide approximates total body-water volume, suggesting that distribution takes place with minimal tissue binding. Various pharmacokinetic studies on ifosfamide have been conducted in both regular patient populations, as depicted in table 2,[34,40,50,63,84-106] and specific patient groups, as depicted in table 3.[107-117]
These studies demonstrated a large effect of autoinduction on the pharmacokinetics of ifosfamide. Clearances observed are difficult to compare, because different approaches were used to calculate this parameter. Paediatric patients are a specific group, due to their distinct pharmacokinetics of ifosfamide. Their differences in body surface area lead to larger variability of ifosfamide doses, making comparison between pharmacokinetic parameters more difficult. Since the volume of distribution also approximates that of body-water, their volumes of distribution are significantly lower than in adults. Ifosfamide can penetrate the blood-brain barrier as concentrations of ifosfamide, 4-hydroxy-ifosfamide and ifosforamide mustard in the cerebrospinal fluid of children were almost equal to concentrations in plasma.[118,119]

Table 2. *Clinical pharmacokinetic parameters of ifosfamide in patients.*

Dose regimen	Patients	$T_{½}$ (h)	$T_{½ini}$ (h)	$T_{½max}$ (h)	V (L)	Cl_{tot} (L/h)	Cl_{ini} (L/h)	Cl_{max} (L/h)	Cl_r (L/h)	UE %	Reference
1.6-2.4 g/m² x3	3	6.9	8.3	6.3							Nelson et al.[84]
5 g/m²/1 h	5	15.2									Allan et al.[85]
2.4 g/m²/1 h x 3	3	6.9									
0.5 g po					48.4	8.9				4.3	McNiel et al.[86]
1.5 g iv					37.0	6.7				9.4	
1.8 g/m² x5	10		8.6	4.8							Piazza et al.[87]
1 g/d	7	5.9			30.1	3.6					Cerny et al.[88]
2 g/d	7	5.3			32.2	4.3					
12-18 g/m²/120 h	27					4.24					Cerny et al.[89]

6 g/m²/72 h	9				25.6	5.1					Pearcey et al.[90]
5 g/m²/0.5 h	6	5.85			31.5	3.9					Lewis et al.[91]
1.5 g/m²/0.5 h x 5	15		7.2	4.6	44.8		4.0	6.9			Lewis et al.[40]
5 g/m²/0.5 h	6	5.4			27.2	3.60					Lewis et al.[92]
5 g/m²/24 h	4	4.5			39.4	4.74					
1.5 g/m²/0.5 h x5	7 cy 1		7.0	3.9	43.8		3.85	7.27	0.40		Lewis et al.[93]
	7 cy 2		7.6	3.9	44.5		3.55	7.02	0.48		
1 g/m² po	12	5.89				5.18					Wagner et al.[89]
1 g/m² iv		5.76				5.02					
2 g/m² po	6	5.71				5.92					
2 g/m² iv		5.33				6.20					
1.5 g/m²/1 h x5	10 iv		6.2	3.8	39.4		4.15	7.39	0.85	2.6	Lind et al.[34,50]
	10 po		5.8	3.5	23.7		3.08	5.15	0.67		
1.2 g/m² x5	7		6.8	3.9	20.4		2.4	4.2		17.2	Benvenuto et al.[95]
1.5 g/m²/1 h x5	11		6.4	4.1						13.9	Kurowski et al.[63,96]
5 g/m² at 41.8°C	6	5.78				8.02					Wiedermann et al.[97]
6-9 g/m²/72 h	21									19	Hartley et al.[98]
4.2 g/1 h (r/s)	12	6.57			42.7	4.55					Corlett et al.[99]
5 g/m²/24 h	15	4.68			39.1	6.02			0.71	14.4	Boddy et al.[100]
7.5-15 g/m²/240 h	22			7.6	58.5		4.76	9.78	0.72	8.5	Kaijser et al.[101]
Various (r/s)	10					ER					Kaijser et al.[102]
3 g/m²/3 h (r/s)	14	6.58				4.84			1.33		Granvil et al.[103]
3 g/m²/3 h (r/s)	11									8.7r 7.2s	Wainer et al.[104]
1.8 g/m² x5	8		5.26	3.58	46.2		5.96	13.9			Comandone et al.[105]
6 g/m²/48 h/1 h x2	11									23.0	Singer et al.[106]

If parameter was specified in kg or m² and average weight or body surface area were give, an average weight of 70 kg and an average body surface area of 1.73 m² was used to recalculate. $T_{½}$=elimination half-life of ifosfamide, $T_{½ini}$=initial $T_{½}$ $T_{½max}$=maximum $T_{½}$ V=volume of distribution of ifosfamide, Cl_{tot}=total clearance of ifosfamide, Cl_{ini}=initial clearance, Cl_{max}=maximum clearance, Cl_r=renal clearance, UE=urinary excretion of unmetabolized ifosfamide dose, (r/s)=pharmacokinetic assessment of enantiomers of ifosfamide, cy=cycle, ER=enantiomeric ratios

Table 3. *Clinical pharmacokinetic parameters of ifosfamide in obese, elderly or paediatric patients.*

Dose regimen	Population	Patients	$T_{½}$ (h)	V (L)	Cl_{tot} (L/h)	Cl_{ini}	Cl_{max}	Cl_r (L/h)	UE %	Reference
1.5 g/m²/0.5 h	obese	4	6.36	42.8	4.56					Lind et al.[107]
x5	normal	12	4.95	33.7	4.33					
1.5 g/m²/0.5 h	>60 y	11	6.03	40	4.83			0.65		Lind et al.[108]
x5	< 60 y	9	3.85	38	4.47			0.60		
6 g/m²/120 h	Paediatric	12	5.2	18.8		3.20	6.79			Passe et al.[109]
9 g/m²/72 h	Paediatric	16	2.12	12.2	4.10			0.54	13.7	Boddy et al.[110]
9 g/m²/72 h	Paediatric	17	2.1		4.43				6.4	Boddy et al.[111]
3 g/m²/1h x3	Paediatric	17	3.2		3.12	3.12	5.20		6.2	
9 g/m²/72 h	Paediatric	11 cy 1	2.06	18.2	5.21					Boddy et al.[112]
		11 cy 2	3.10	27.2	5.40					
9 g/m²/72 h	Paediatric	21				3.27	7.50			Boddy et al.[113]
0.4-3 g/m²	Paediatric	22							23.0	Silies et al.[114]
0.4-3 g/m²	Paediatric	14							56.0r 44.0s	Boos et al.[115]
2 g/m²/1 h x3 3 g/m²/3 h x3 9 g/m²/72 h	Paediatric	5	3.63			4.49	6.25			Prasad et al.[116]
2.8 g/m²/1 h	Paediatric			30	2.4					Ducharme et al.[117]

$T_{½}$=elimination half-life of ifosfamide, V=volume of distribution of ifosfamide, Cl_{tot}=total clearance of ifosfamide, Cl_{ini}=initial clearance, Cl_{max}=maximum clearance, Cl_r=renal clearance, UE=urinary excretion of unmetabolized ifosfamide dose, (r/s)=pharmacokinetic assessment of enantiomers of ifosfamide

Administration

Various ways of administration of ifosfamide and their impact on the metabolism and toxicities have been studied. The pharmacokinetic differences observed after fractionation of the administered ifosfamide dose seem to be largely caused by differences in autoinduction.[34,40,92,93] Autoinduction was typically seen between day 1 and day 5, but was always reset to initial values at the start of a consecutive course.[93] However, a recent study did not demonstrate any differences in ifosfamide or metabolite areas under the concentration-time curve (AUC) between bolus and continuous ifosfamide infusion.[106] In studies with continuously infused ifosfamide, autoinduction was maximal after three to 10 days.[101] As the decrease in elimination half-life of ifosfamide could not be explained by an increase in renal clearance, a metabolic difference was implicated. A shift towards more hepatic metabolism was observed by monitoring the dechloroethyl and 4-hydroxyifosfamide metabolites.[63,110] No preference in shift was observed between activation and deactivation. Oral versus intravenous infusion of ifosfamide has also been compared. Oral bioavailability of ifosfamide was close to 100%, but oral administration resulted in unacceptable neurotoxicity. This may be due to a shift in metabolism towards dechloroethylation compared to intravenous infusion.[34,94,120] Subcutaneous admini-stration and portable pumps were also successful for outpatient treatment.[101,121,122] High-dose regimens are of special interest since high-dose chemotherapy should be more effective due to the steep dose-response relationship observed in pre-clinical models[123] and clinical studies.[124] The AUC appeared to increase in

a linear fashion with increasing doses (up to at least 16 g/m²) of continuously administered intravenous ifosfamide, but saturation was observed in doses over 16 g/m².[89] Saturation was observed only in ifosforamide mustard and not ifosfamide. Since data showed wide interpatient variability and the bioanalytical method used had a relatively large reproducibility error, thus far saturation has not been demonstrated conclusively.

Chirality

Ifosfamide is a chiral drug with an asymmetric phosphorus atom and is always administrated as a racemic mixture.[125] The last decade, various clinical studies have been conducted to estimate the effect of this chirality on the pharmacokinetics of ifosfamide and its metabolites.[99,102-104,114-117] Urinary excretion of unchanged (R)-ifosfamide was significantly higher than (S)-ifosfamide, what could be due to different affinities for CYP of the enantiomers. (S)-ifosfamide could also preferentially be converted to the dechloroethyl metabolites. The chirality for the dechloroethylation route during induction by phenobarbital, resulted in a specific metabolic shift, possibly due to interspecies differences in CYP isoenzymes in rats compared to humans.[126] A difference was also found in human liver microsomes.[23,24] In theory, pure (R)-ifosfamide administration in humans could lead to less development of neurotoxicity, since (R)-ifosfamide is less dechloroethylated and more activated than (S)-ifosfamide, but the clinical relevance needs to be investigated in controlled clinical trials.

Variability

Large interpatient variability in clinical toxicity and response rates have been observed in patients treated with ifosfamide[124], which may be explained by differences in pharmacokinetics of ifosfamide. Interindividual variations in drug disposition can be caused by genetic or environmental factors.[127] Genetic factors are rather easily identifiable, such as (over)expression of specific metabolic enzymes. For instance, the presence of CYP3A in pulmonary carcinomas may lead to local activation of ifosfamide in the tumour, which may result *in situ* cytotoxic activity.[128] CYP3A upregulation has been identified in several tumour types.[128] Intratumoural CYP3A4 may therefore be a predictive marker for the effectiveness of ifosfamide therapy.[21] Another co-variate for ifosfamide pharmacokinetic variability is age. Elderly non-small cell lung cancer patients demonstrated doubling of elimination half-life, apparently due to an increased volume of distribution. Total body, renal and nonrenal clearance were unaltered.[108] Obesity also doubled the elimination half-life, due to an increased volume of distribution.[107] Renal impairment has few implications for ifosfamide disposition, since renal clearance is only minor.[129] However, renal insufficiency might increase the risk of neurotoxicity, due to more extensive deactivation of ifosfamide as a result of decreased renal excretion of ifosfamide. Dose reduction in patients with renal failure was suggested.[130] Ifosfamide metabolism is mainly hepatic indicating an possible effect of liver impairment. Liver impairment is known to slow the metabolism of ifosfamide without shifting the metabolization towards more activation or deactivation.[131] Complete hepatic failure resulted in a total blockage of metabolism and first-order (renal) pharmacokinetics of

ifosfamide.[132] Further, insights into factors influencing ifosfamide pharmacokinetics may lead to improved dosing of ifosfamide and thus improve the anti-tumour efficacy and toxicity of ifosfamide treatment.

Modelling

The observed variability in pharmacokinetic parameters also arise from application of different approaches in pharmacokinetic evaluations. During the last decades several methods have been proposed for the description of the pharmacokinetics of ifosfamide and its metabolites.[109] Allen et al. and Lind et al. used a two-compartment pharmacokinetic model.[85,34] Nelson et al. proved a one-compartment pharmacokinetic model to be superior for lower doses but not for higher doses.[84] Boddy et al. used a model independent approach for describing ifosfamide pharmacokinetics.[110] These methods were developed to describe concentration-time profiles of infusions of short duration (1-3 hours) and did not take into account the effects of autoinduction on the pharmacokinetics of ifosfamide. Prasad et al. and Boddy et al. were the first to report models, which enabled more adequate description of the concentration-time data for ifosfamide infusions of longer duration of up to 72 hours.[116,112] Their models included a lag time before the development of autoinduction and described the increase of clearance of ifosfamide over time. Recently, a new model was published that simultaneously described both cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics using a population approach. This mechanism-based model used a hypothetical enzyme compartment to estimate the effects of autoinduction on the pharmacokinetics of cyclophosphamide and its metabolite.[131] Recently, a similar approach was used for ifosfamide.[132] This population pharmacokinetic model did not require a lag time to describe the concentration-time profile of ifosfamide. A population approach is preferred to single-subject pharmacokinetics, because it will allow assessment of interindividual and residual variability, necessary for therapeutic drug monitoring.

Pharmacodynamics

Cytotoxicity

The cytotoxic effect of ifosfamide is believed to be caused by its ultimate alkylating metabolite, ifosforamide mustard. After intracellular activation, the two chloroethyl-groups of ifosforamide mustard will be converted to reactive electrophilic alkyl-groups ($R\text{-}CH_2^+$), which in turn react with the nucleophilic moieties of the bases in DNA, such as *N*-7-guanine. Due to its bi-functional character, ifosforamide mustard is able to form two reactive alkylgroups. Attachment of these alkylgroups to two bases can result in an intrastrand link, if the two bases are in the same DNA strand, or in an interstrand cross-link or if the two bases are on different DNA strands. Especially, the latter will impair DNA replication by inhibition of double-strand separation prior to cell division.[134] Impairment of function of duplex DNA will result in cytotoxicity. Since tumour cells have a higher rate of cellular divisions, they will be affected at a higher rate than normal cells by this cytotoxic effect. Ifosforamide mustard is an analogue of

the phosphoramide mustard of cyclophosphamide. In contrast to phosphoramide mustard, which has two exocyclic 2-chloroethylgroups, ifosforamide mustard has one 2-chloroethyl group on the exo- and one on the endocyclic oxazaphosphorine nitrogen atom.[136] This apparently provides a more effective DNA cross-linking distance between the two independent functional alkylating moieties. DNA cross-linking will be an increasingly important pharmacodynamic endpoint in clinical trials. Cross-linking may possess a more direct relationship to clinical outcome like toxicity and efficacy than pharmacokinetics. Recent development of analytical methods for assessing DNA cross-linking enabled thorough pharmacodynamic monitoring of chemotherapeutic trials.[137] Irreparable cross-linking of DNA strands, depurination and chain-scission can induce permanent alterations in the DNA structure, resulting in mutagenesis and carcinogenesis. A second mechanism of anti-tumour activity has been proposed as chloroacetaldehyde-induced depletion of intracellular sulfhydryl agents, such as gluthatione. Normal gluthatione levels have been shown to protect DNA against alkylation by reacting with ifosforamide mustard.[138] For instance, cytotoxicity was increased in lymphocytes after gluthatione depletion.[139] Furthermore, gluthatione regulated multi-drug resistance could be overcome by chloroacetaldehyde-induced depletion.[140] These findings were confirmed by the *in vitro* cytotoxicity of chloroacetaldehyde observed in human stem cells, solid tumour cells and leukaemia cell lines.[141,142] Beside gluthatione depletion, inhibition of aldehyde dehydrogenase could also increase cytotoxicity (or overcome multi-drug resistance), because it can metabolize 4-hydroxyifosfamide to inactive compounds.[138] A third cytotoxic effect could be achieved by acrolein. Acrolein was able to cause single-strand DNA breaks, which possibly adds to the cytotoxic effects.[143] Co-administration of mesna may, however, prevent this cytotoxic effect.

Efficacy

Ifosfamide therapy has anti-tumour activity against small and non-small cell lung carcinoma, testicular tumours, soft tissue sarcomas and osteosarcomas, breast cancer, ovarian cancer, cervix carcinomas, malignant lymphomas, acute leukaemias, multiple myelomas, plasma cell leukaemia head and neck cancer, melanoma, prostatic cancer, renal cell carcinoma, bladder cancer and gastrointestinal tumours. Ifosfamide is also active against a wide range of paediatric tumours, such as rhabdomyosarcoma, soft tissue sarcoma, Ewing sarcoma and osteosarcoma, neuroblastoma, Wilms' tumour, childhood leukaemia and paediatric lymphomas. Anti-tumour activity of ifosfamide is reached in both single agent and combination therapy with many other cytotoxic agents, such as platinum compounds, vinca alkaloids, etoposide, mitomycin and adriamycin. Efficacy expressed as tumour response, time to relapse and survival rates vary strongly dependent on tumour type, pre-treatment and dosing. Due to its single agent activity ifosfamide has been added to various combination regimens. Combination therapy aims at additive or synergistic non-cross-resistant efficacy with non-overlapping toxicity, based upon different mechanisms of action. The wide range of malignant diseases in which ifosfamide is effective is markedly different from its isomer, cyclophosphamide, due to differences in pharmacology. The difference in the rate of metabolic transformation results in different clinical pharmacokinetic profiles for the two final alkylating mustard metabolites. The extensive inactivation by dechloroethylation of ifosfamide

is responsible for the fact that ifosfamide is dosed three to tenfold higher than cyclophosphamide.[144,145] This also explains the more neurotoxic character of ifosfamide compared to cyclophosphamide. However, in comparison to phosphoramide mustard, ifosforamide mustard was able to alkylate DNA with a higher affinity and had its effects over a longer period of time, due to the stereochemical orientation of its alkylating moieties.[146]

Haematological toxicity

The principal dose-limiting toxicity of ifosfamide is myelosuppression, especially leukocytopenia. White blood cell nadirs usually occur between days eight to 13 of the treatment cycle. Haematological recovery in most patient groups will usually be complete by day 17 or 18 of the treatment cycle, facilitating chemotherapeutic retreatment in four week cycles. Recovery can be accelerated by treatment with cytokines, e.g. granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor or interleukine-3.[147] The incidence of leukocytopenia is dose-related[148] and fractionated dosage schedules of ifosfamide tend to produce less myelosuppression than single high-dose schedules.[149] A white blood cell count below 3 x 10^9/L is expected in approximately 50% of patients treated with single agent ifosfamide treatment at doses of 1.2 g/m²/day for 5 days, while at this dose level thrombocytopenia with platelets below 100 x 10^9/L can be expected in about 20% of patients.[149]

Neurotoxicity

Encephalopathy

Central neurotoxicity is the non-haematological dose-limiting toxicity of oral and high-dose ifosfamide treatment. Encephalopathy caused by ifosfamide is characterized by confusion, sleepiness, drowsiness, hallucinations, amnesia and clear dreams.[150,151] Epileptic insults and coma have also been described.[152] Changes in the electro-encephalogram of patients suffering from ifosfamide-induced encephalopathy have been reported.[153,154] Although in most cases encephalopathy was only temporary and reversible, deadly outcome has been reported mainly in children.[155-158] Acute symptoms have been observed between two and 96 hours after the start of ifosfamide administration, but reversed within 48 to 72 hours after cessation of administration.[159,155] Onset of acute symptoms after 12 to 146 hours and a duration of 3 days has also been described.[160] Some long-term sometimes irreversible effects have been reported such as mild symptoms of emotional instability, apathy, short-term memory problems and mental focusing problems.[160]

Encephalopathy mechanisms

Various predisposing factors exist for ifosfamide-induced encephalopathy. Factors identified were oral ifosfamide therapy, decreased hepatic and renal function, previous chemotherapy with cisplatin, low serum albumin levels, pelvic abnormalities (obstruction of tractus urogenitalis) and brain metastases (irradiation of central nervous system). A higher incidence was also observed in women and elderly patients.[161,162] The irreversible renal toxicity caused by cisplatin predisposed for ifosfamide-induced encephalopathy and not the exposure

to cisplatin itself.[155,163] Drug-drug interaction may also lead to an altered ifosfamide metabolism through enzyme induction or competition. Oral therapy and short duration intravenous infusions have a higher risk of encephalopathy than continuous infusions, suggesting a relation with maximal plasma concentrations. Most predisposing factors could be correlated to clearance of ifosfamide and its metabolites. For instance, renal insufficiency could decrease unchanged ifosfamide excretion, possibly resulting in more extensive metabolism. Mesna which is always co-administered with ifosfamide therapy, does not cause encephalopathy when administered as single agent.[164] No differences were observed in pharmacokinetic parameters, such as elimination half-life and clearance of excreted unchanged ifosfamide between patients with and without encephalopathy.[165] Thus it was proposed that, an abnormal high concentration of a specific metabolite of ifosfamide would be the most likely candidate to cause encephalopathy and not the alkylating activity of ifosfamide in the cerebrospinal fluid as was suggested earlier.[166,167] Cyclophosphamide does not exhibit neurotoxicity. Thus, metabolic differences between ifosfamide and cyclophosphamide can yield further insights into the mechanism. For the greater part cyclophosphamide is metabolized by ring-oxidation yielding 4-hydroxycyclophosphamide and phosphoramide mustard and only 1-2% of cyclophosphamide is dechloroethylated. In contrast, ifosfamide is metabolized for 25-60% by dechloroethylation, which results in approximately a tenfold increase of chloroacetaldehyde concentrations.[159] Oral administration of ifosfamide results in a metabolic shift towards increased dechloroethylation compared to intravenous administration. Moreover, ifosfamide is administered at 10 times higher doses than cyclophosphamide, which gives rise to a hundredfold increase of chloroacetaldehyde concentrations. Chloroacetaldehyde and structural closely related metabolites of ethanol (acetaldehyde) and the hypnotic chloral hydrate (trichloroacetalde-hyde) all exhibit central nervous activity.[168] Chloroacetaldehyde serum concentrations in two paediatric patients suffering from encephalopathy were markedly increased, compared to four asymptomatic paediatric patients. These findings suggest induction of encephalopathy by chloro-acetaldehyde and not by dechloroethyl metabolites, as was proposed earlier.[169] Only few clinical pharmacokinetic studies have been performed on chloroacetaldehyde, because of bioanalytical stability problems. Chloroacetaldehyde has an elimination half-life of only 2-3 minutes, which necessitates stabilization directly after sample collection.[170] Furthermore, due to its rapid elimination half-life, caution must be taken in extrapolating serum concentrations of chloroacetaldehyde to cerebrospinal fluid concentrations, which will have the best relationship with the site of action. As chloroacetaldehyde is formed equimolarly with each dechloroethyl metabolite of ifosfamide, pharmacokinetics of 2-dechloroethyl-ifosfamide and 3-dechloro-ethylifosfamide could provide further insights into the risk of central neurotoxicity. Elevated concentrations of 2-dechloroethylifosfamide and 3-dechloroethyl-ifosfamide were observed in patients with encephalopathy receiving a 10-day continuous infusion of 1500-1750 mg/m² ifosfamide.[101] Chloroacetaldehyde (and 4-hydroxyifosfamide) cause a rapid depletion of gluthatione in lymphocytes, both *in vivo* and *in vitro*.[139] Gluthatione depletion in the brain could also result in neurotoxicity because detoxification is impaired.[171] Acetylcysteine administration could theoretically normalize the gluthatione depletion by acting as a gluthatione precursor. Cerebral gluthatione depletion in mice treated with ifosfamide and CYP3A enzyme inducers phenobarbital or dexamethason was correlated with the severity of

neurotoxic symptoms.[172] The gluthatione regeneration capacity of the liver is much higher than that of the brain. This elucidates the fact that hepatotoxicity has a lower incidence than encephalopathy. Beside gluthatione depletion in the brain, inhibition acetyl-CoA-dehydrogenase dependent processes in the mitochondrial terminal respiration chain by chloroacetaldehyde may result in neurotoxicity. Several studies have demonstrated elevated levels of urinary excreted glutaric acid and sarcosine in patients with ifosfamide-induced encephalopathy.[173] These observations are similar to symptoms seen in glutaricaciduria type II, a syndrome caused by a shortage of electron-transporting flavoproteins (type I) or a shortage of acetyl-CoA-dehydrogenases (type II). The latter enzyme is essential in the regeneration of reduced acetyl-CoA in the mitochondrial terminal respiration chain. Reduced acetyl-CoA is unable to transfer electrons. This will result in an accumulation of toxic intermediaries of the Krebs-cyclus possibly resulting in neurotoxicity.[162] Therefore, chloroacetaldehyde may interfere with the oxidative phosphorylation in the mitochondria and thereby disturbing the acetyl-CoA-dehydrogenase dependent processes.

Treatment of encephalopathy

Methylene-blue (methylthionine) has been used successfully in the treatment of glutaricaciduria type II. The mechanism of action of methylene-blue is based upon its ability to act as a non-physiological electron-acceptor, thereby regenerating the reduced acetyl-CoA. Methylene-blue was also able to compensate the chloroacetaldehyde effect on the long-chain fatty acid oxidation, thus preventing encephalopathy and fatigue.[174] Methylene-blue inhibits several mono-amino-oxidases *in vitro*. Besides hepatic CYP dependent metabolism of ifosfamide, extrahepatic oxidation of ifosfamide in the mitochondria in the brain by these mono-amino-oxidases may also lead to chloroacetaldehyde. Prophylactic methylene-blue may therefore not only counter the effect of chloroacetaldehyde, but may also prevent its formation.[171,175] If encephalopathy (CTC-grade II/III) is observed due to ifosfamide, cessation of infusion, regaining a correct electrolyte balance, use of neuroleptics or benzodiazepines for symptom fighting (exclusion of brain metastases is essential) and use of methylene-blue may be indicated. Haemodialysis has also been successful in reversing neurotoxicity.[129] Although few randomized studies have been conducted, several case reports have shown that encephalopathy can be treated successfully and prevented with 50 mg methylene-blue p.o. every 4 hours in a 5%-glucose solution. Treatment must continue until full recovery is observed.[162,173,176,177] Glucose solution is used because analogous to glutaricaciduria type II treatment glucose compensates for the disturbed fatty acid oxidation and decreased gluconeogenesis. One randomized study with effective methylene-blue treatment showed that the pharmacokinetics and metabolism of ifosfamide were not altered.[178]

Urotoxicity

During the middle of the 1960's urotoxicity was the dose-limiting toxicity of ifosfamide, which often manifested as haemorraghic cystitis. Urotoxicity can be prevented by co-administration of mesna as it reacts specifically with acrolein in the urinary tract.[9] Specificity of the detoxification-reaction is based upon the pH-dependent moieties of mesna.[179,180] After oral or intravenous administration the thiol-compound mesna is rapidly auto-oxidized in plasma at

pH 7.4 to dimesna, which is inactive. Dimesna is highly hydrophilic resulting in a poor tissue distribution and a rapid renal clearance, but also in prevention of penetration of tumour cell membranes. Hence, no impairment of cytotoxicity of ifosfamide can be expected.[181,182] In the renal tubular epithelial dimesna is converted back to mesna by gluthatione reductase enzymes. The free sulfhydryl groups of mesna will react with the double bond of the urotoxic acrolein or with the potentially urotoxic 4-hydroxyoxazaphosphorine metabolites to form stable and non-toxic compounds. In contrast to acrolein-mesna, 4-hydroxyoxazaphosphorine-mesna has been detected in urine. From this it was concluded that the main anti-urotoxicity activity of mesna is prevention of acrolein formation from 4-hydroxyoxazaphosphorines rather than derivatization of acrolein itself.[75] In order to maintain optimal urothelial protection, mesna should be given continuously before, during and after ifosfamide administration.[183] Outdoor patient oral administration of mesna proved to be equally effective against urotoxicity of ifosfamide if patient compliance is sufficient.[184,185] Some concern has been raised over co-administration of platinum agents and mesna (e.g. cis- and carboplatin), because *in vitro* cell lines were protected from platinum cytotoxicity by mesna.[186] Since these agents are frequently administered in combination chemotherapy with ifosfamide, a decreased efficacy of the platinum agents may be expected.

Nephrotoxicity

Another non-haematological toxicity of ifosfamide is its nephrotoxicity. Especially children are at risk. Predictive risk factors are age, cumulative ifosfamide dose, concurrent administration of cis- or carboplatin, unilateral nephrectomy and ifosfamide administration.[187] Ifosfamide-induced nephrotoxicity may lead to Fanconi Syndrome (FS) which has been reported in over 60 cases, predominantly in paediatric populations. Incidence ranged from 1.4 to 5% of all ifosfamide-treated patients. Fanconi Syndrome is a global impairment of the proximal tubule function including glucose, protein, phosphate, bicarbonate and amino acid transport. Fanconi Syndrome is generally irreversible, long-lasting and potentially progressive. Damage clinically manifested itself as polyuria, metabolic acidosis and renal phosphate wasting, resulting in hypophosphatemia induced rickets. Phosphate suppletion and vitamin D may then be indicated.[188] Another 15% of patients developed subclinical tubular dysfunction.[189] Renal dysfunction can be tested by determining urinary retinol binding protein or ß2-microglobulin.[190] The cause of ifosfamide-induced Fanconi Syndrome is unclear. Cyclophosphamide does not cause nephrotoxicity. Extensive dechloroethylation is only observed with ifosfamide. Chloroacetaldehyde has been mentioned as possible causative of Fanconi Syndrome.[191] *In vitro* renal cells have been able to metabolize ifosfamide to 2- and 3-dechloroethylifosfamide, thus locally providing chloroacetaldehyde.[14,192] Furthermore, chloroacetaldehyde binds to carnitine. Carnitine depletion impairs the energy production in mitochondria resulting in a local energetic failure in the proximal tubules.[193,194] However, no clinical studies have shown correlation between systemic production of chloroacetaldehyde and nephrotoxicity.[195] Secondly, a difference in rate and extent of alkylation compared to cyclophosphamide may be a cause. Fanconi Syndrome may also be caused by local gluthatione depletion in the proximal tubules due to recirculating mesna, but this does not explain the difference in the development of nephrotoxicity between ifosfamide and

cyclophosphamide.[196,197]

Miscellaneous toxicities

Alopecia occurs in almost all patients treated with ifosfamide.[198] Nausea and/or vomiting can occur within a few hours of ifosfamide administration and incidence is dose-related. Standard anti-emetic drugs administered prior or simultaneously with ifosfamide can control these symptoms.[199] Hypersensitivity to ifosfamide is rare but has been reported, consisting of redness in the face, left arm, back and chest and bronchoconstriction.[200] Case-reports mentioned cardiotoxicity and interstitial pneumonitis but no predisposing factors were identified.[201,202]

Pharmacokinetics-pharmacodynamics

During the development of ifosfamide therapy assessment of pharmacokinetic-pharmacodynamic relationships was not part of the standard drug development as it is now.[203] Ifosfamide development focused on identification of metabolites and their roles in efficacy and toxicity. Lack of reliable bioanalytical methodology has long hampered these attempts. Thus far, only some ambiguous pharmacokinetic-pharmacodynamic relationships of ifosfamide and its metabolites were identified. Since ifosfamide is a prodrug, no correlation between plasma levels of the parent drug and toxicity and/or response was expected. In contrast, it has been demonstrated that increased cyclophosphamide clearance resulted in less relapse but increased risk of cardiac toxicity.[204,205] Increased clearance of cyclophosphamide may have lead to increased formation of activated metabolite. Ifosfamide demonstrated a relationship between the area under the plasma alkylating activity curve and the nadir leukocyte count.[206] Furthermore, high ifosfamide concentrations were related to renal toxicity.[207] Markedly, a negative relationship has been found between both progression free interval and survival, and ifosforamide mustard area under the plasma curve.[100] In the same study a positive correlation of urinary recovery of the dechloroethylated metabolites of ifosfamide was found with survival.[100] Both findings are in contrast with the assumed relationship of ifosforamide mustard and ifosfamide efficacy and the assumption of lack of activity of 2- and 3-dechloroethylifosfamide. Possibly plasma alkylating activity as determined with thin layer chromatography does not necessarily reflect intratumoural DNA alkylation. Furthermore, the equimolarly formed chloroacetaldehyde could well posses anti-tumour activity.[140] Prolonged low exposure of 4-hydroxycyclophosphamide was more efficacious than short infusions in an animal model.[208] Thus far, the complex process of activation and detoxification of ifosfamide, lack of reliable bioanalytical methodology and its infrequent use as single agent, hampered the definition of unambiguous pharmacokinetic-pharmacodynamic relationships between systemic exposure of the relevant metabolites and their anticipated beneficial or toxic effects.[209]

Since high dose therapy is generally close to the maximum tolerated dose, the concept of pharmacokinetic variability is very important. Wide interpatient pharmacokinetic or exposure variability could result in sub-therapeutic treatment and at the same time in possibly lethal

overdosing. By choosing a population approach in quantifying the pharmacokinetics of ifosfamide, the observed large interpatient variability could be taken into account. A second step should be the identification of co-variables correlating with abnormal pharmacokinetic profiles. Then correlations between the pharmacokinetics of chloroacetaldehyde or the equimolar 2- and 3-dechloroethylifosfamide and the neurotoxicity of ifosfamide have to be made. Also, activated ifosfamide metabolites either 4-hydroxyifosfamide or ifosforamide mustard must be correlated with clinical efficacy and haematological toxicity. Recently, even toxicity was used to evaluate efficacy by correlating leukocyte nadir with distant disease-free survival.[210,211] By combining all pharmacokinetic-pharmacodynamic relationships, including variability on both pharmacokinetic and pharmacodynamic population parameters, therapeutic drug monitoring of ifosfamide may become essential. Therapeutic drug monitoring can be done after assessment of the pharmacokinetics of a first course of ifosfamide followed by a dose alteration in subsequent courses tailored to the patient. Secondly, therapeutic drug monitoring could be achieved a priori by assessing specific co-variables correlating with pharmacokinetics or pharmacodynamics. Both methods require an extensive database of pharmacokinetic and pharmacodynamic data with a wide range of variability.

Conclusions

Although ifosfamide has successfully been used for over 30 years in the treatment of various malignant diseases, there is still a need for a mechanism-based understanding of the autoinducible metabolism, transport through the body, pharmacokinetics and its variability, cytotoxicity, neurotoxicity, urotoxicity and nephrotoxicity of ifosfamide. The lack of high-throughput validated bioanalytical methods has long been the limiting factor in ifosfamide research. Now that these methods have finally been developed, the underlying mechanisms of above mentioned phenomena will be better understood and a superior ifosfamide therapy can be developed, better tailored to the individual patient.

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Chapter 2.1

Determination of ifosfamide, 2- and 3-dechloroethylifosfamide using gas chromatography with nitrogen-phosphorous or mass spectrometry detection

Summary

A comparison was made between methods for determining ifosfamide, 2- and 3-dechloroethylifosfamide using gas chromatography with nitrogen-phosphorous detection (GC-NPD) versus positive ion electron-impact ion-trap mass spectrometry (GC-MS^2). Sample pretreatment involved liquid-liquid extraction with ethyl acetate after adding trofosfamide as internal standard and alkalinization. The GC-NPD was linear, specific and sensitive for all analytes in the range of 0.0500-100 µg/ml with lower limits of quantification (LLQ) of 0.0500 µg/ml using a 50-µl plasma sample. The GC-MS^2 was linear specific, and sensitive for ifosfamide, 2- and 3-dechloroethylifosfamide in the ranges of 0.250-100, 0.500-25.0 and 0.500-25.0 µg/ml, respectively, with LLQs of 0.250, 0.500 and 0.500 µg/ml. The ranges of accuracy, within-day precision and between-day precision for analysis of all compounds with GC-NPD did not exceed 93.3-105.4%, 8.0% and 9.8%, respectively. The ranges of accuracy, within-day precision and between-day precision for analysis of all compounds with GC-MS^2 did not exceed 86.5-99.0%, 9.0% and 12.7%, respectively. In conclusion, GC-NPD proved to be superior to GC-MS^2 in sensitivity, detection range, accuracy and precisions. Therefore, GC-NPD is the method of choice for fast underivatized determination of ifosfamide, 2- and 3-dechloroethyl-ifosfamide in human plasma and can readily be used for clinical pharmacokinetic studies and routine monitoring of ifosfamide treated patients in a hospital setting.

Introduction

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide (Holoxan®, Ifex®), is commonly used to treat various solid tumours, soft tissue sarcomas and hematological malignancies in adults and children. Ifosfamide is a member of the oxazaphosphorine class of cytotoxic drugs along with cyclophosphamide and trofosfamide, as shown in figure 1.[1]

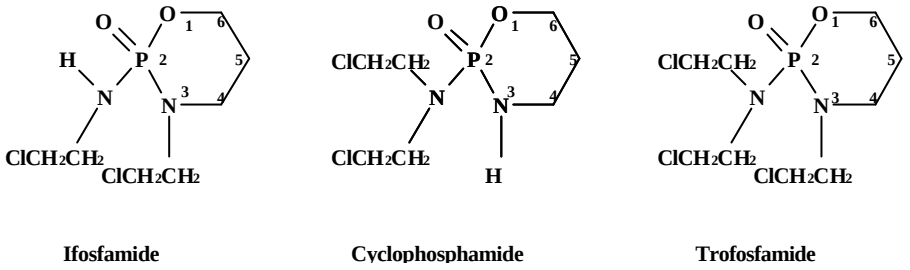

Figure 1. *Structures of ifosfamide, cyclophosphamide and trofosfamide.*

Ifosfamide is a prodrug which needs activation via the cytochrome P450 enzymatic system in the liver.[2] It is also subject to deactivation routes that involve removal of a chloroethylgroup from either the exo- or endocyclic nitrogen atom to form 2- and 3-dechloroethylifosfamide, respectively. An equimolar amount of the neurotoxic chloroacetaldehyde is formed, as represented in figure 2.[3]

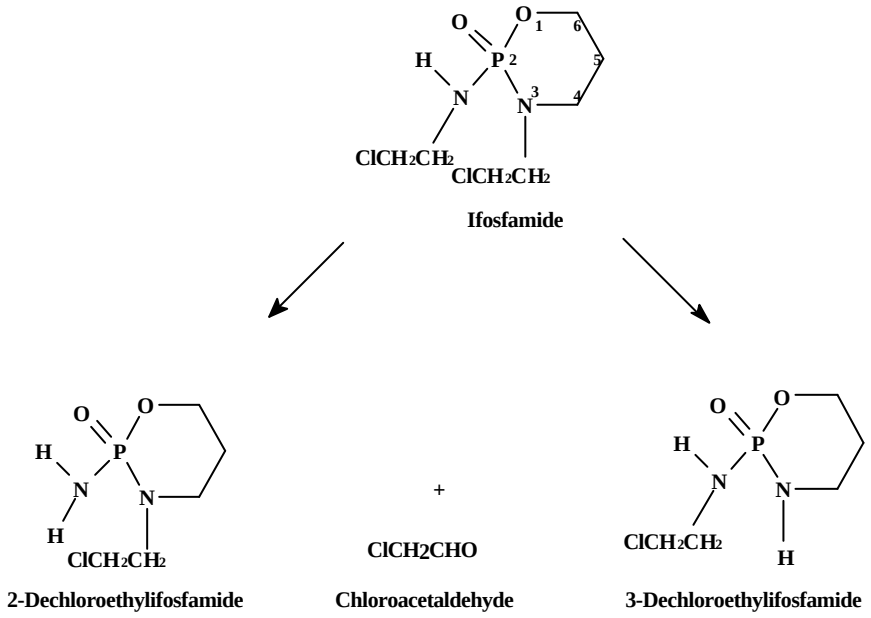

Figure 2. *Dechloroethylation of ifosfamide.*

Ifosfamide is a chiral compound. Possible chiral differences are suspected, but their effect on the efficacy and toxicity of ifosfamide is not clear. Central neurotoxicity is the dose-limiting toxicity of high-dose ifosfamide treatment and can vary from mild hallucinations to coma and death.[4] Hence, 2- and 3-dechloroethylifosfamide pharmacokinetics are crucial for insight into the risk of central neurotoxicity during ifosfamide treatment. By monitoring these metabolites during ifosfamide treatment, dose adjustments could be suggested for following treatment courses. Modern, bioanalytical methods require high sample-throughput, accuracy and precision in a clinically relevant concentration range, when applied in clinical pharmacokinetic studies. To support our clinical studies, we aimed to develop a fast, new, direct method that fulfilled these criteria for monitoring ifosfamide, 2- and 3-dechloroethylifosfamide using gas chromatography. In the past various bioanalytical methods have been reported for quantifying ifosfamide and its dechloroethylated metabolites. Thin layer chromatography with various detection methods is easy to apply, but is hampered by cross-selectivity and low sensitivity.[5] A more elaborate and expensive method is nuclear magnetic resonance (NMR) spectroscopy, which has been used to identify new metabolites of oxazaphosphorines.[6] High-performance liquid chromatography is easy to use but because of its undiscriminating detection at lower wavelengths (200-300 nm), labour-intensive sample preparation is required to separate the analytes from interfering plasma components.[7] Previously published enantiomer-selective methods were less robust and high sample-throughput was not feasible.[8,9] In the last decade the use of bioanalytical methods with mass spectrometric (MS) detection has increased enormously. These techniques are usually preferred due to theoretical increased selectivity.[10] To justify the choice for detection we compared ion-trap mass spectrometry (GC-MS^2) with a more conventional method using gas chromatography with nitrogen-phosphorous selective detection (GC-NPD), without the use of a derivatization agent.[11,12]

Materials and methods

Chemicals

Ifosfamide, 2- and 3-dechloroethylifosfamide and trofosfamide were kind gifts of Dr J. Pohl, ASTA Medica AG (Frankfurt, Germany). Ethyl acetate (p.a. grade) was obtained from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

Instrumentation

A gas chromatograph (HP 5890 series II, Hewlett Packard, Amstelveen, The Netherlands) equipped with a split/splitless injector, a model HP 7360 autosampler (Hewlett Packard, Amstelveen, The Netherlands) and a flame ionization nitrogen-phosphorous selective detection system (NPD) were used. Separation was achieved on a capillary CP Sil 8 CB 25 m $\times$ 0.25 mm column coated with 5% phenyl-groups and 95% dimethylpolysiloxane-groups

with a film thickness of 0.25 µm (Chrompack, Middelburg, The Netherlands). Data were recorded with a Spectra Physics model 4270 integrator (Spectra Physics, Santa Clara, CA, USA) and reprocessed using the PC1000 system (Thermo Separation Products, Breda, The Netherlands). A combined Finnigan MAT GCQ gas chromatograph/mass spectrometer with an ion-trap (Thermo Quest, Breda, The Netherlands) was used with a split/splitless injector and a model A200S autosampler (Thermo Quest, Breda, The Netherlands). Detection was achieved in positive ion electron-impact mode followed by parent-daughter fragmentation (EI-MS^2) with mass selective detection. Separation was achieved on a capillary DB-5MS low-bleed 30 m x 0.25 mm column, also coated with 5% phenyl-groups 95% dimethylpolysiloxane-groups with a film thickness of 0.25 µm (J&W Scientific, Folsom, CA, USA). The characteristics of the analytical columns used in both systems were identical, but the DB-5MS was specifically adapted for low-bleed MS^2-applications. Data were analyzed using GCQ software on a Gateway 2000 P5-75 personal computer (Gateway, NJ, USA).

Chromatographic conditions

Both the injector and detector temperatures of the GC-NPD system were 275°C. A straight inlet-liner with a funnel-shaped collection chamber (without glasswool) was installed. Split flow was set at 1:10. Helium was used as a carrier gas at a flow-rate of 3.0 ml/min. The flow-rates of the detection gases hydrogen, air and auxiliary (helium) were 2.6, 95 and 25 ml/min, respectively. The starting temperature of the column oven (200°C) was maintained for 7 min. and then ramped to 225°C at 40°C/min. and kept at 225°C for 2.88 min., resulting in a total run time of 10.5 min. The injector temperature of the GC-MS^2 system was 275°C, the transfer line temperature was 275°C and the ion source temperature was 200°C. A straight inlet-liner (without glasswool) was installed. Split flow was set at 1:10. The column flow had a constant linear velocity of 40.00 cm/sec. The oven temperature programme started at 120°C and was ramped directly to 225°C at 20°C/min. and kept at 225°C for 9.75 min, resulting in a total run time of 15.00 min. MS-detection was performed in positive ion electron-impact mode with an ion current of 70 eV, an ion-trap and refragmentation with a collision energy 0.8 V. From 0 to 5.00 min., the MS detector was switched off. From 5.00 to 8.00 min. daughter-fragments at m/z 149.0 were refragmented yielding the quantification-fragments for 2- and 3-dechloroethylifosfamide at m/z 56.0. From 8.00 to 10.00 min. daughter-fragments at m/z 211.0 were refragmented yielding the quantification-fragment for ifosfamide at m/z 70.0. From 10.00 to 15.00 min. daughter-fragments at m/z 273.0 were refragmented yielding the quantification-fragment for trofosfamide at m/z 118.0.

Calibration standards and quality control samples

Samples for the standard calibration curves were obtained by adding ifosfamide, 2- and 3-dechloroethylifosfamide solutions in distilled water to 50 µl drug-free human plasma in a 1.5-ml Eppendorf cup. The stock solutions were prepared fresh prior to the preparation of calibration and quality control samples. The resulting concentrations of the standard calibration curve for each of the analytes were 0, 0.0500, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 µg/ml. A final volume of 100 µl was obtained by adding water. A volume of 50 µl of a 2.5 µg/ml trofosfamide solution in distilled water, 10 µl of a 0.5 M sodium hydroxide solution and 500 µl of ethyl acetate was then added. The solutions were whirlmixed for 30 sec. and centrifuged for 10 min. at 3,000 g at ambient temperature. Extraction recoveries from plasma for ifosfamide, 2- and 3-dechloroethylifosfamide of 86, 44 and 69% have been reported.[11,12] There is a lower extraction recovery of 2-dechloroethylifosfamide, because it has a higher polarity than 3-dechloroethylifosfamide. A 450-µl volume of the organic layer was transferred to a 1.5 ml Eppendorf cup and evaporated under a gentle stream of nitrogen at 40°C. No degradation was observed for the analytes compared to ambient temperature evaporation. Operating at 40°C sped up the evaporation step. The residue was reconstituted in 25 µl ethyl acetate. The injected volume in the GC-NPD and GC-MS^2 was 1 µl. Quality control samples, containing 0.0500, 0.100, 0.250, 10.0 and 100 µg/ml ifosfamide, 2- and 3-dechloroethylifosfamide in plasma were prepared independently from the standard calibration curve as described above. Five replicate quality control samples of each concentration were analyzed simultaneously with the calibration curves in three different analytical runs on both the GC-NPD and GC-MS^2 systems.

Selectivity and specificity

Interference by endogenous compounds was investigated by analysis of six different blank plasma samples. Regular co-medication of ifosfamide treatment and other anti-cancer drugs (granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, MESNA, ketoconazole, caffeine, topotecan, paclitaxel, etoposide, cisplatin and carboplatin) were investigated for interference with the analytical methods.

Limit of quantification

The lower limit of quantification (LLQ) was investigated by determining five quality controls at the LLQ in three analytical runs. Each analytical run was made with plasma from a different donor. For the concentration to be accepted as the LLQ, the percentage of deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) were required to be less than 20%. The upper limit of quantification (ULQ) was defined as 100 µg/ml in accordance with expected clinical concentrations.

Accuracy, precisions and linearity

Accuracy, between-day precision and within-day precision of the methods were determined by assaying five replicate standard samples in plasma at five different concentrations (0.0500,

0.100, 0.250, 10.0 and 100 µg/ml) in three different analytical runs. Accuracy was measured as the percentage of the nominal concentration. The within-day and between-day precisions were obtained by analysis of variance for each test concentration using the analytical run as a grouping variable. For the construction of each calibration curve 12 spiked plasma samples were analyzed in duplicate. Optimization of their weighting factors was investigated. Linearity of the three plasma calibration curves was tested with the *F*-test for lack of fit.

Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *p*-values were 0.05 or less.

Results and discussion

Chromatography

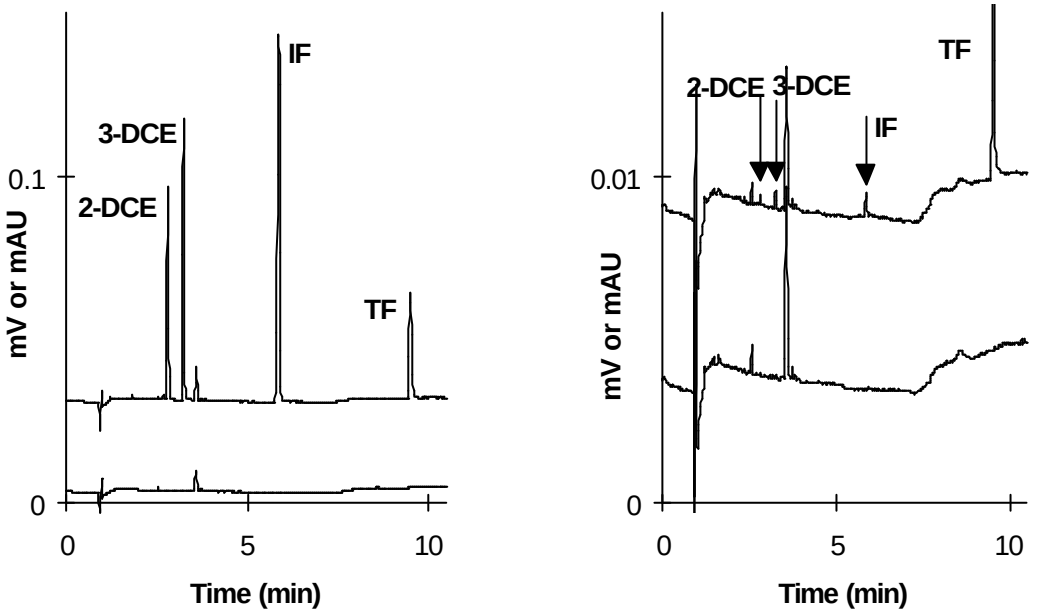

Figure 3. Left panel: *Typical chromatogram by gas chromatography with nitrogen-phosphorous detection of a plasma sample spiked with 10.0 µg/ml 2- (2-DCE) and 3-dechloroethylifosfamide (3-DCE) and ifosfamide (IF), with trofosfamide (TF) as internal standard (top) and blank plasma (bottom).* Right panel: *Typical chromatogram of a plasma sample spiked with 0.100 µg/ml 2-DCE, 3-DCE and IF with TF as internal standard (top) and blank plasma (bottom).*

The GC-NPD chromatogram of a 10.0 and a 0.100 µg/ml spiked plasma sample in figure 3 demonstrated retention times of 2.8, 3.3, 5.9 and 9.5 min. for 2- and 3-dechloroethyl-ifosfamide, ifosfamide and trofosfamide, respectively. The peak with a retention time of 3.6 min. was identified as caffeine. The GC-MS^2 chromatogram in figure 4 demonstrated retention times of 6.0, 6.5, 8.6 and 12.7 min. for 2- and 3-dechloroethylifosfamide, ifosfamide and trofosfamide, respectively.

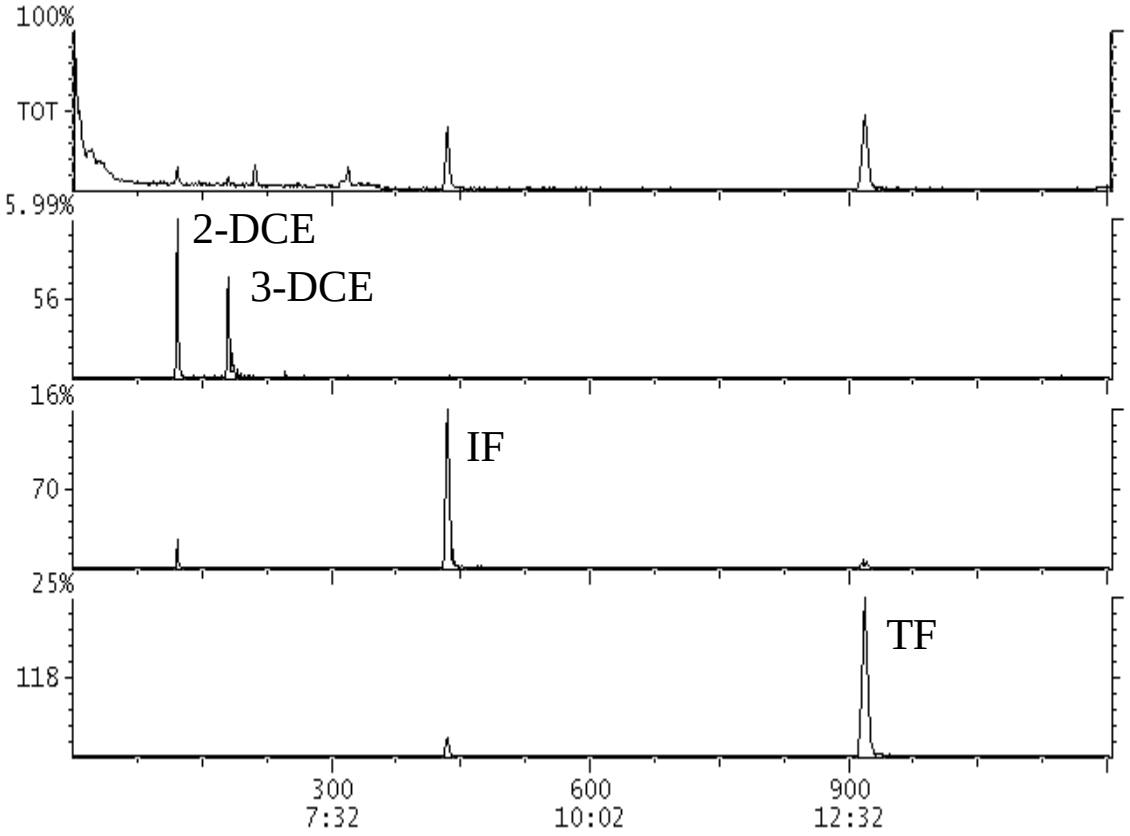

Figure 4. *Typical chromatogram by gas chromatography with mass spectrometry with daughter-ion fragmentation of a plasma sample spiked with 10.0 µg/ml 2- (2-DCE) and 3-dechloroethylifosfamide (3-DCE) and ifosfamide (IF), with trofosfamide (TF) as internal standard.* From top to bottom: *total ion-current, 2-DCE and 3-DCE quantification-fragments at m/z 56, IF quantification-fragment at m/z 70 and TF quantification-fragment at m/z 118*

Detection

The NPD system used a rubidium-salt detector with a high sensitivity for nitrogen and phosphorous atoms producing a high selectivity for ifosfamide and its metabolites. GC-MS^2 operated with selected reaction monitoring (SRM) in which the analyte (parent ion) is fragmented and a specific daughter-ion is selected in the ion-trap and subsequently refragmented. The mass spectra observed are represented in figure 5. Ifosfamide has a molecular weight (Mw) of 261.1 g/mol.

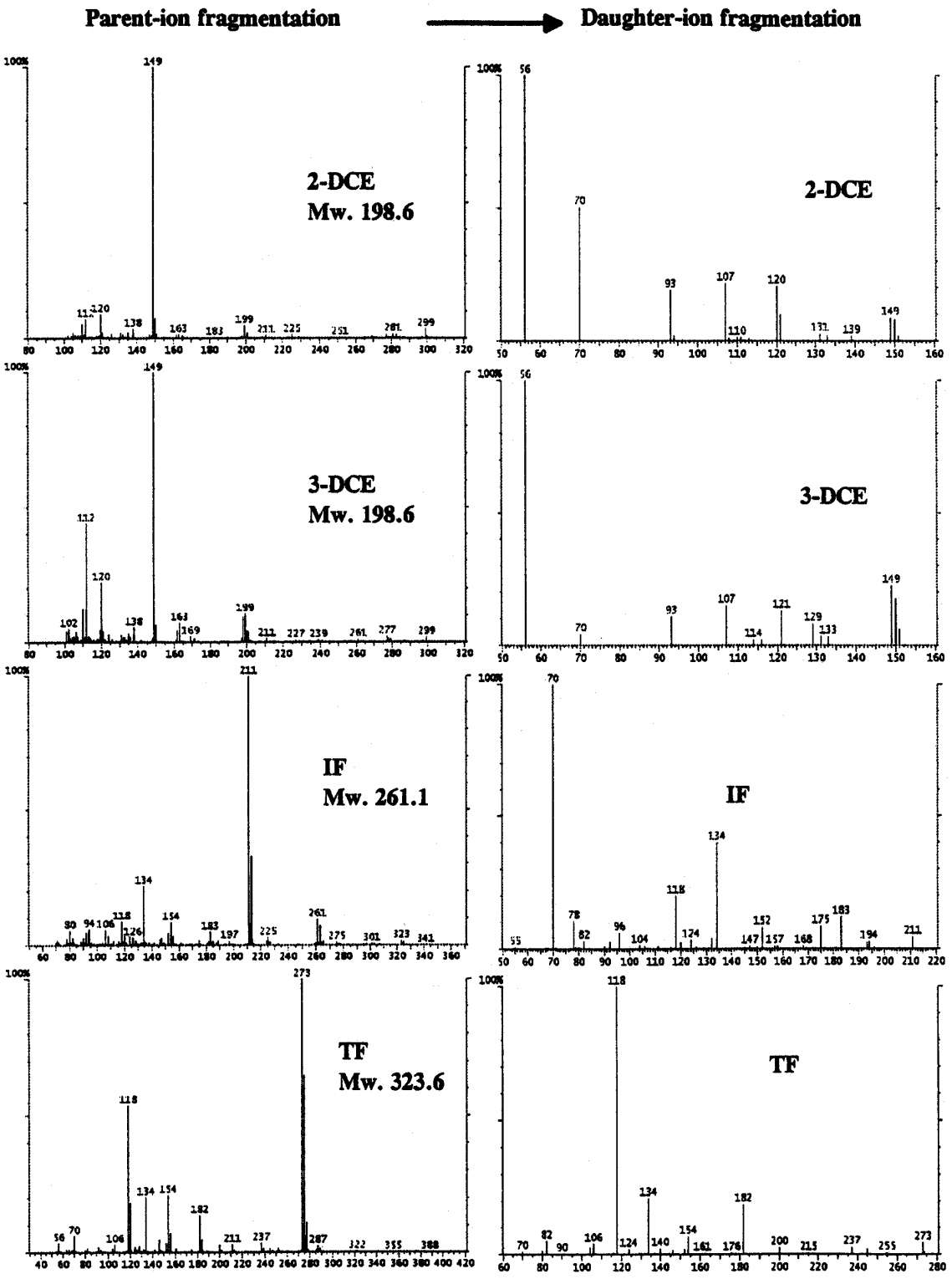

Figure 5. *Mass-spectra of 2- (2-DCE) and 3-dechloroethylifosfamide (3-DCE), ifosfamide (IF) and trofosfamide (TF) fragmentation. Refragmentation of daughter fragments at m/z 149, m/z 149, m/z 211 and m/z 273, respectively yielded quantification-fragments at m/z 56, m/z 56, m/z 70 and m/z 118, respectively.*

After EI^+ the parent-ion (M^+) with mass-to-charge ratio (m/z) of 261 was fragmented. The base peak at m/z 211 with its typical isotope peak at m/z 213 contains 1 Cl-atom. The base peak of ifosfamide at m/z 211 can be explained by a loss of a CH_3Cl. CH_3Cl could be split off from either the exo- or endocyclic chloroethylgroup of ifosfamide. The isomers 2- and 3-dechloroethyl-ifosfamide with Mw 198.6 yielded M^+s with m/z 199. Fragmentation lead to the base peak at m/z 149, due to a loss of CH_3Cl. No M^+ peak was observed with trofosfamide. Trofosfamide has a Mw 323.6. The base peak m/z 273 with two isotope peaks, could be explained by a loss of CH_3Cl. For all analytes the M-CH_3Cl fragment was selected for further fragmentation. Daughter-ion fragmentation of the ifosfamide M-CH_3Cl fragment (m/z 211) yielded a base peak at m/z 70, which could be explained by $(CH_2NHCH_2CHCH_2)^+$. Daughter-ion fragmentation of the 2- and 3-dechloroethyl-ifosfamide M-CH_3Cl fragments (m/z 149) yielded both a base peak at m/z 56, which could be explained by $(CH_2NHCHCH_2)^+$. Daughter-ion fragmentation of the trofosfamide M-CH_3Cl fragment (m/z 118) yielded a base peak at m/z 118, which could be explained by $NPNHCH_2CH_2CH_2O$. In theory, the selectivity of the SRM mode used, should be much greater than that obtained in single ion monitoring (SIM) mode. To produce a false positive result, the interfering compound must fragment to a daughter-fragment of the same m/z and refragment identically to a quantification-fragment of the same m/z. This advantage of SRM over SIM resulted in a relatively higher sensitivity due to increased selectivity (data not reported). A major disadvantage of SRM, however, is the lower absolute sensitivity, because refragmentation results in wasting mass. Due to the second fragmentation only 40, 48, 40 and 54% of the daughter-fragments of ifosfamide, 2- and 3-dechloroethyl-ifosfamide and trofosfamide was retreived as final quantification-fragment. Therefore, although very selective the GC-MS^2 system was inappropriate for sensitive determination of 2- and 3-dechloroethylifosfamide. Furthermore, non-linearity in response was observed at concentrations higher than 25 µg/ml. This resulted in underestimated responses and non-linear calibration curves at higher concentrations. This non-linearity could possibly be explained by column-overloading or non-linearity in detector response.

Specificity and selectivity

After sample pretreatment with liquid-liquid extraction, plasma samples spiked with granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, MESNA, ketoconazole, topotecan, paclitaxel, etoposide, cisplatin and carboplatin did not display any interference with either the GC-NPD or GC-MS^2 method. Caffeine was detected by GC-NPD but did not interfere due to its retention time of 3.6 min. Blank plasma samples of six different individuals showed no interfering endogenous substances. A typical chromatogram of blank spiked plasma by GC-NPD is shown in figure 3.

Limit of quantification

The LLQs using GC-NPD for all analytes were 0.0500 µg/ml each. The percentages of deviation from the nominal concentration of ifosfamide, 2- and 3-dechloroethylifosfamide by GC-NPD at these concentrations were less than 15.5%, 19.4% and 18.2%, respectively. The relative standard deviations of ifosfamide, 2- and 3-dechloroethyl-ifosfamide with GC-NPD were less than 5.7%, 5.3% and 5.7%, respectively. The LLQs using GC-MS^2 for ifosfamide, 2- and 3-dechloroethylifosfamide were 0.250, 0.500 and 0.500 µg/ml, respectively. Because of the insufficient sensitivity of the GC-MS^2 system, the LLQ concentrations of 2- and 3-dechloroethylifosfamide could not be tested using the quality control samples prepared. The percentage of deviation from the nominal concentration of ifosfamide using GC-MS^2 was less than 11.4%. The relative standard deviation of ifosfamide using GC-MS^2 was less than 9.8%. Thus, 0.0500 µg/ml was defined as the LLQ for ifosfamide, 2- and 3-dechloroethylifosfamide using GC-NPD and 0.250, 0.500 and 0.500 µg/ml using GC-MS^2, respectively.

Accuracy, precisions and linearity

The use of the weighting factor of $1/(conc.)^2$ resulted in a minimal sum of deviations from the nominal concentrations, and prevented the relative standard deviations of the duplicates and the deviations from the nominal concentrations from exceeding 15% or, in case of the LLQ, 20%. Removal of the weighting factor and using a weighting of 1/conc. both resulted in deviations from the nominal concentration for the LLQ of greater than 30%. This could possibly be explained by non-linearity in detection or column-overloading. Intercepts, slopes and correlation coefficients (r^2) describing the calibration curves of all analytes measured using GC-NPD and GC-MS^2 are represented in table 1.

Table 1. *Standard calibration curves of ifosfamide (IF), 2- (2DCE) and 3-dechloroethyl-ifosfamide (3DCE) for validation of GC-NPD and GC-MS^2 (n=3).*

Analyte	Method	Range (µg/ml)	Slope±S.D.	Intercept±S.D.	Correlation coefficient±S.D.
IF	NPD	0.0500-100	0.4073±0.0220	0.0024±0.0009	0.9986±0.0003
2DCE	NPD	0.0500-100	0.0824±0.0029	-0.0002±0.0006	0.9992±0.0007
3DCE	NPD	0.0500-100	0.1448±0.0126	0.0004±0.0014	0.9975±0.0012
IF	MS^2	0.250-100	0.1471±0.0072	0.0102±0.0009	0.9954±0.0017
2DCE	MS^2	0.500-25.0	0.0350±0.0134	-0.0010±0.0014	0.9945±0.0016
3DCE	MS^2	0.500-25.0	0.0331±0.0151	-0.0054±0.0025	0.9901±0.0062

Abbreviations: S.D.= standard deviation

Correlation coefficients determined using GC-NPD and GC-MS^2 were always greater than 0.995 and 0.990, respectively, as determined by least squares analysis. All calibration curves by GC-NPD were linear in the range of 0.0500-100 µg/ml with use of the *F*-test for lack of fit. Ifosfamide, 2- and 3-dechloroethylifosfamide determined by GC-MS^2 were linear only for ranges of 0.250-100, 0.500-25.0 and 0.500-25.0 µg/ml. GC-NPD was more accurate and

more precise (within-day and between-day) than GC-MS^2 in the concentration ranges specified for ifosfamide, 2- and 3-dechloroethylifosfamide, as represented in tables 2 and 3. In addition to being superior to GC-MS^2 in accuracy and precisions, GC-NPD used a less expensive detection system.

Table 2. *Accuracy, within-day and between-day precisions of ifosfamide (IF), 2- (2DCE) and 3-dechloroethylifosfamide (3DCE) analysis in human plasma determined by GC-NPD, calculated using one-way ANOVA (p<0.05) (n=5).*

Analyte	Concentrations	Accuracy (%)		Precision (%)	
	(µg/ml)	Mean ± S.D.	95% C.I.	Within-day	Between-day
IF	0.0500	103.2 ± 6.5	99.6-106.8	5.5	3.5
IF	0.100	99.9 ± 2.5	98.5-101.3	0.8	2.9
IF	0.250	104.5 ± 3.7	102.4-106.6	1.8	3.6
IF	10.0	102.0 ± 3.0	100.3-103.7	2.0	2.6
IF	100	93.3 ± 4.8	90.6-95.6	2.9	5.1
2DCE	0.0500	103.2±7.7	98.9-107.4	5.1	6.4
2DCE	0.100	98.9±9.0	94.0-103.9	3.8	9.8
2DCE	0.250	96.4±5.5	93.4-99.5	3.2	5.7
2DCE	10.0	100.4±1.9	99.3-101.5	1.9	0.4
2DCE	100	100.6±2.5	99.3-102.0	2.1	1.6
3DCE	0.0500	103.6±8.7	98-9-108.4	5.1	7.8
3DCE	0.100	93.6±9.3	88.5-98.8	8.0	6.8
3DCE	0.250	97.9±6.8	94.1-101.6	5.0	5.7
3DCE	10.0	105.4±6.1	102.0-108.8	4.5	4.4
3DCE	100	102.5±5.5	99.5-105.6	3.0	5.3

Abbreviations: S.D.= standard deviation, C.I.= confidence interval.

Table 3. *Accuracy, within-day and between-day precisions of ifosfamide (IF), 2- (2DCE) and 3-dechloroethylifosfamide (3DCE) analysis in human plasma determined by GC-MS^2, calculated using one-way ANOVA (p<0.05) (n=5).*

Analyte	Concentrations	Accuracy (%)		Precision (%)	
	(µg/ml)	Mean ± S.D.	95% C.I.	Within-day	Between-day
IF	0.250	94.6±9.0	89.6-99.6	7.8	6.4
IF	10.0	99.0±11.0	92.9-105.1	2.9	12.7
IF	100	86.5±5.7	83.3-89.7	3.8	6.4
2DCE	10.0	95.9±9.0	90.9-100.9	8.0	5.7
3DCE	10.0	96.3±11.0	90.2-102.4	9.0	8.4

Abbreviations: S.D.= standard deviation, C.I.= confidence interval.

Analysis of patient samples

The applicability of the selected GC-NPD assay for pharmacokinetic studies in patients was demonstrated, as depicted in figure 6. The assay allowed monitoring of plasma levels of ifosfamide, 2- and 3-dechloroethylifosfamide in a patient receiving 1.5 g/m² ifosfamide in 1 hour for two days.

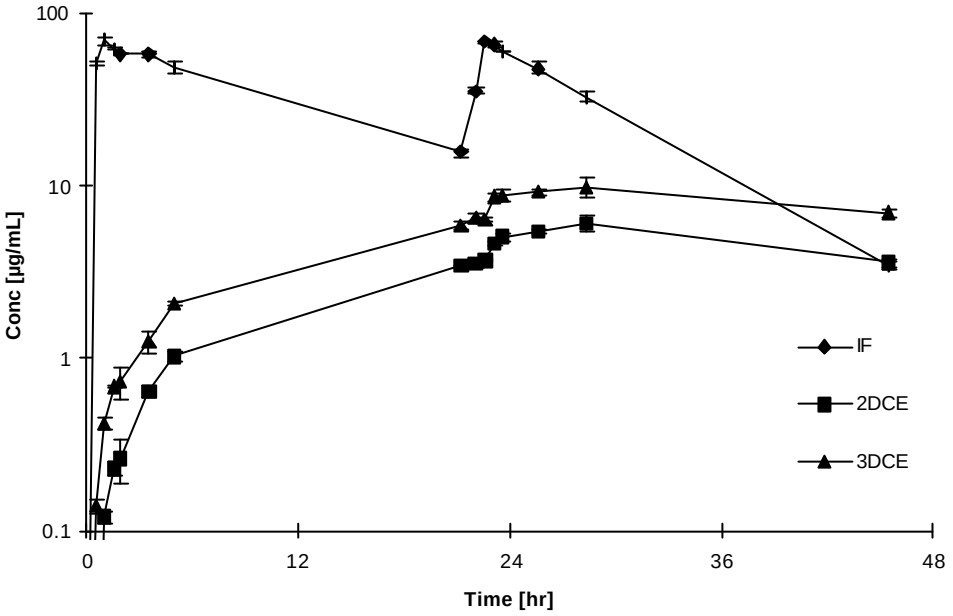

Figure 6. *Plasma concentrations of ifosfamide (IF), 2- (2DCE) and 3-dechloroethylifosfamide (3DCE) in a patient receiving 1.5 g/m² ifosfamide in 1 hour for two days.*

Conclusions

Two GC methods for quantification of underivatized ifosfamide, 2- and 3-dechloroethyl-ifosfamide were compared. The more conventional method using GC-NPD proved to have a better detection range, accuracy and precisions than a recently developed method using GC-MS^2. The GC-NPD assay meets the current requirements for the validation of a bioanalytical methodology and can be used for pharmacokinetic studies of ifosfamide treated patients. It is our experience that the described GC-NPD assay can readily be used in a hospital laboratory environment for routine monitoring of ifosfamide, 2- and 3-dechloroethylifosfamide concentrations. Thus far, we have analyzed more than 5,000 samples without major problems.

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Chapter 2.2

High-performance liquid chromatographic determination of stabilized 4-hydroxyifosfamide in human plasma and erythrocytes

Summary

A method using reversed-phase high-performance liquid chromatography is described for the measurement of the stabilized activated metabolite of ifosfamide, 4-hydroxyifosfamide, in human plasma and erythrocytes. Immediately after sample collection and plasma-erythrocyte separation at 4°C, 4-hydroxyifosfamide was stabilized by derivatization with semicarbazide. The sample pretreatment involves liquid-liquid extraction with ethyl acetate. Reversed-phase high-performance liquid chromatography was executed with a C8 column and acetonitrile-0.025 M potassium dihydrogen phosphate buffer (pH 7.40)-triethylamine (13.5:86:0.5 v/v) as mobile phase. The analyte was determined with UV-detection at 230 nm. Complete validation, optimization and stability studies were performed and the method proved to be specific, sensitive and with a stable analyte in the range of clinically relevant concentrations (0.1-10 µg/ml) after conventional dosing. The lower limit of quantitation was 100 ng/ml using a 1.00 ml of sample. Accuracy was between 94.1 and 107.0%. Within-day and between-day precisions were less than 6.2% and 7.2%, respectively. 4-Hydroxyifosfamide-semicarbazide was found to be stable in the biological matrix at -20°C for at least 1 month. A pharmacokinetic study conducted in a patient receiving 9 g/m^2 in 3 days by means of a continuous infusion demonstrated the applicability of this method.

Introduction

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine 2-oxide (Holoxan®, Ifex®), is commonly used for the treatment of various solid tumours, soft tissue sarcomas and haematological malignancies in adults and children. Ifosfamide is a prodrug which needs activation via the cytochrome P450 enzymatic system in the liver to 4-hydroxyifosfamide. 4-Hydroxyifosfamide exists in equilibrium with its tautomeric form aldoifosfamide. Aldoifosfamide can be converted into the final alkylating agent ifosforamide mustard with concurrent acrolein formation. Since 4-hydroxyifosfamide/aldo-ifosfamide is the activated form of the prodrug ifosfamide and because 4-hydroxy-ifosfamide/aldoifosfamide in contrast with ifosforamide mustard is capable of penetrating a (tumour)cell, systemic 4-hydroxyifosfamide/aldoifosfamide concentrations may yield the most relevant and easily accessible reflection of a concentration-therapeutic effect correlation. Erythrocytes are of special interest, because it was proposed that they act as transporters of 4-hydroxyifosfamide and 4-hydroxycyclophosphamide the activated metabolite of cyclophosphamide, an isomer of ifosfamide.[1,2]

Various laborious methods for the determination of 4hydroxyifosfamide in whole blood, plasma and erythrocytes have been developed. An indirect method widely used is based on the release of acrolein from 4-hydroxyifosfamide, followed by trapping with 3-aminophenol yielding 7-hydroxyquinoline which is amenable to fluorometric analysis.[3,4] The acrolein that is generated in vivo could interfere with this method, but due to the unstable character of acrolein in blood this is not likely. Another technique applies thin-layer chromatography combined with fluorometric detection.[5] The lower limit of detection with this method is 1 mg/ml and therefore this method is not useful for patient plasma analysis using small volume samples. Furthermore, no stability data were presented. An elaborate method using gas chromatography with mass spectrometric detection was also developed but its use of self-synthesized deuterium labelled analogues as internal standards renders it less favourable. This method stabilizes 4-hydroxyifosfamide to a cyanohydrin adduct, followed by dichloromethane extraction and silyl derivatization.[6] New methods were developed using ^{31}P nuclear magnetic resonance (NMR) [7] and ^{1}H-NMR-high-performance liquid chromatography.[8] Thus far, no relatively simple, repro-ducible validated method using high-performance liquid chromatography has been developed. The highest hurdle in the analysis of 4-hydroxyifosfamide is its instability in biological fluids. However, by adding semicarbazide the tautomeric equilibrium of 4-hydroxyifosfamide/aldo-ifosfamide is stabilized with subsequent formation of a stable semicarbazone derivative, as depicted in figure 1. This prevents spontaneous decomposition to ifosforamide mustard.[9] An analogous method for the quantitative analysis of 4-hydroxycyclophosphamide was published recently.[10]

Our aim was to develop a novel selective and sensitive high-performance liquid chromatographic method for 4-hydroxyifosfamide analysis in biological samples, which offers sufficient robustness to investigate 4-hydroxyifosfamide pharmacokinetics in patients receiving high dose ifosfamide treatment. The mentioned requirements can be met by stabilizing 4-hydroxyifosfamide with semicarbazide and by using the resulting semicarbazone derivative for high-performance liquid chromatographic analysis.

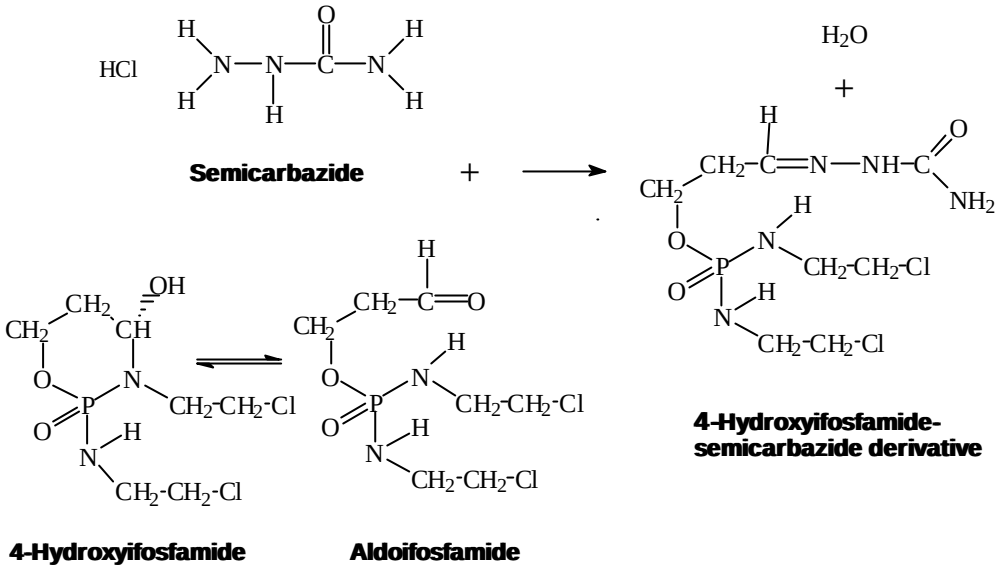

Figure 1. *Stabilization of 4-hydroxyifosfamide by reaction of semicarbazide with the tautomeric aldoifosfamide yielding the derivative 4-hydroxyifosfamide-semicarbazone.*

Materials and methods

Equipment

The high-performance liquid chromatographic system consisted of a Thermo Separation Products (TSP) Spectraseries System with a model AS3000 automated injector with a 100 µl loop and a model P1000 pump connected to a model UV1000 detector with ultra-violet detection at 230 nm. Data were collected by the TSP system and transferred by model SN4000 analog-digital convertor to a Dell optiplex Gs Pentium personal computer. A 5-µm Merck Hibar Lichrosorb C8 RP-Select B (25 cm x 4.0 mm I.D.) column was equipped with a 10-µm Chrompack pre-column.

Chemicals

4-Hydroperoxyifosfamide (Lot 033.6496) and all other ifosfamide and cyclophosphamide metabolites were a kind gift of Dr J Pohl, ASTA Medica AG (Frankfurt, Germany). Acetonitrile and ethanol (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Ethyl acetate (p.a. grade), triethylamine (p.s. grade) and anhydrous potassium dihydrogen phosphate (suprapure grade) were obtained from Merck (Darmstadt, Germany). Semicarbazide hydrochloride (p.a. grade) was purchased from Acros (Geel, Belgium). A 2 M semicarbazide solution was made and adjusted to pH 7.40 with 1 M HCl and 1 M NaOH, prior to use. Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands). Blank erythrocytes were collected from volunteers in the Slotervaart Hospital (Amsterdam, The Netherlands).

Preparation of standards

4-Hydroperoxyifosfamide was used as a substitute for 4-hydroxyifosfamide. In aqueous solution, 4-hydroperoxyifosfamide rapidly liberates 4-hydroxyifosfamide/aldoifosfamide and hydrogen peroxide. Fresh solutions of 4-hydroxyifosfamide were prepared immediately prior to use by dissolving an equimolar amount of 4-hydroperoxyifosfamide in distilled water. Dissolution was accelerated by ultra-sonification. Standard concentration curves were obtained by adding 1 ml drug-free plasma of 4°C to known amounts of unstabilized 4-hydroxyifosfamide in the range 0, 0.10, 0.25, 0.50, 1.0, 5.0 and 10.0 µg/ml in 5 ml polypropylene reaction tubes. Immediately after vortexing for 15 s. 100 µl 2 M semicarbazide (pH 7.40) was added and vortexed rigorously for 1 min.

Patient sample collection

Venous blood samples of a patient receiving intravenous ifosfamide treatment (9 g/m^2/72 hours) were taken at 0, 3, 10, 20, 24, 48, 68, 72, 73, 76, 80 hours after start of the infusion. The samples were collected in 5 ml polypropylene reaction tubes and immediately cooled by placing them in ice water. The samples were then centrifuged without delay for 5 min. with 1,000 g at 4°C. Two 1 ml plasma samples were collected and added to 100 µl 2 M semicarbazide (pH 7.40). The left-over plasma was collected for ifosfamide analysis according to the method as described by Kaijser et al.[11] The white blood cell layer was discarded with sufficient margins. 1 ml erythrocytes was collected from the erythrocyte-layer and added to 100 µl 2 M semicarbazide (pH 7.40). The left-over erythrocytes were also collected for ifosfamide analysis. All semicarbazide-stabilized samples were vortexed rigorously for 1 min. and stored at -70°C pending analysis. The sample collection and stabilization procedure was performed within a 10 min. time-period.

Sample pretreatment

After thawing 3 ml ethyl acetate was added to all patient and standard samples. After vortexing for 1 min. the samples were centrifuged for 10 min. with 1,000 g. The organic layer was separated and evaporated to dryness under a gentle nitrogen flow at 40°C. The samples were reconstituted in 150 µl mobile phase. Total reconstitution was achieved by ultra-sonificating the sample for 15 min. followed by vortexing for 1 min. The injected volume into the high-performance liquid chromatography system was 100 µl.

Chromatography

The mobile phase consisted of acetonitrile-0.025 M potassium dihydrogen phosphate buffer-triethylamine (13.5:86:0.5 v/v) adjusted to pH 7.40 with 2 M hydrochloric acid. The UV wavelength for detection was 230 nm. The flow-rate was 0.8 ml/min. The analytical column was operated at ambient temperature.

Mass spectrometric identification of 4-hydroxyifosfamide-semicarbazide

A solution containing 5.7 mg 4-hydroxyifosfamide was derivatized with 9.2 mg semicarbazide (an approximately four times equimolar semicarbazide amount). After ex-traction with ethyl acetate the derivative was evaporated to dryness. Also, a 1-ml aliquot of eluted 4-hydroxyifosfamide-semicarbazide was collected after high-performance liquid chromatographic analysis and extracted with ethyl acetate. The residues were recon-stituted in a 50% acetonitrile solution and injected into an electron spray VG Platform (Fissons instrument) electron impact mass spectrometry system for identification.

Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank plasma and erythrocytes samples. The following related compounds were investigated for interference with the analytical method: ifosfamide, 4-ketoifosfamide, carboxyifosfamide, 2-dechloroethylifosfamide, 3-dechloroethylifosfamide, ifosforamide mustard and 4-hydroxycyclophosphamide-semicarbazide. Possible co-medication was also tested for interference. Tested substances were granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, topotecan and sodium 2-mercaptoethane sulphonate (MESNA).

Limit of quantification and limit of detection

The lower limit of quantification (LLQ) was investigated in plasma samples from three different donors, by a five-fold determination in three analytical runs. For the concentration to be accepted as the LLQ, the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) were to be less than 20%. The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The upper limit of quantification (ULQ) was arbitrarily defined as 10 µg/ml.

Validation: accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precisions of the method were determined by assaying six replicate standard samples in plasma at four different 4-hydroxyifosfamide concentrations (0.1, 0.5, 5 and 10 µg/ml) in three different analytical runs. These quality control samples were made independent of standard curve concentrations. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and

between-day precision were obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as grouping variable. For the construction of each calibration curve seven spiked plasma samples were analyzed in duplicate. After optimization of their weight factors, linearity of the three plasma and three erythrocyte calibration curves was tested with the *F*-test for lack of fit. Recovery of 4-hydroxy-ifosfamide-semicarbazide after sample pretreatment procedure was determined by comparing observed 4-hydroxyifosfamide-semicarbazide in extracted plasma and erythrocytes samples to those of non-processed standard solutions made of 4-hydroxy-ifosfamide-semicarbazide diluted in mobile phase and direct injection.

Optimization

The possible breakdown of 4-hydroxyifosfamide before stabilization with semicarbazide was determined. This was carried out by exposing standard plasma samples of 0.5 and 5 µg/ml 4-hydroxyifosfamide in duplicate (unprotected by semicarbazide) to 4°C, room temperature and 37°C for 0, 10, 20, 30, 60 and 90 min. Directly after exposure the standards were stabilized with semicarbazide and stored at -20°C pending standard analysis. Optimization of incubation was tested by using standard plasma samples of 0.5 and 5 µg/ml 4-hydroxyifosfamide in triplicate, stabilized with semicarbazide and exposing them to 4°C, room temperature, 40°C and 60°C for 0, 10, 20 and 60 min. The influence of the amount of semicarbazide on the stability and chromatography was tested by adding various amounts of semicarbazide (100 µl of 0.125, 0.25, 0.5, 1.0 and 2.0 M) to standard plasma samples of 0.5 and 5 µg/ml 4-hydroxyifosfamide in duplicate, followed by sample pretreatment as described.

Stability during storage

Stability during storage was tested by storing standard plasma samples of 0.5 and 5 µg/ml 4-hydroxyifosfamide in triplicate, stabilized with semicarbazide, at 37°C and 60°C for one hour and 24 hours, at room temperature for one hour, 24 hours, three days and seven days, and at -20°C and -70°C (including three additional freeze-thawing cycles) for one hour, 24 hours, three days, seven days and one month. After the storage period the samples were treated as described and after organic layer evaporation stored as dry samples at -70°C pending further analysis. Analysis was always done within one month.

Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *p*-values were 0.05 or less.

Results and discussion

Chromatography and detection

A new bioanalytical assay was developed for the determination of 4-hydroxyifosfamide. Recently, a bioanalytical assay was described for 4-hydroxycyclophosphamide.[10] Although some parallels exist between the two methods we did change the chromato-graphy substantially. Furthermore, a detailed optimization and stability study was performed in order to validate every step in the handling of the samples from collection in the clinic to bioanalysis in the laboratory. The spectral analysis of 4-hydroxyifosfamide-semicarbazide displayed a maximal chromophoric activity at 230 nm. The reversed-phase chromatography was investigated at different mixtures of acetonitrile in order to find the optimal combination of good separation and a practicable retention time for the analyte. By raising the pH to 7.40 full separation of interfering peaks and the analyte was achieved. Peak shape was further optimized by addition of 0.5% triethylamine.

Sample pretreatment

For 4-hydroxycyclophosphamide-semicarbazide a two-step liquid-liquid extraction (LLE) with ethyl acetate : chloroform (3:1 v/v) is described in order to separate the analyte from interfering substances.[10] Since no interfering peaks were found for 4-hydroxyifosfamide-semicarbazide with the chromatography as described above, extraction could be reduced to a single step. Furthermore, the extraction efficacy could be increased by using ethyl acetate alone. Optimal balance between extraction of 4-hydroxyifosfamide-semicarbazide and efficient removal of interfering endogenous compounds was found to be at pH 7.40. Alkalinization to pH 12 resulted in a higher extraction efficacy, but this coincided with increased levels of interfering substances. Since 4-hydroxycyclophos-phamide-semicarbazide is also detectable its usefulness as an internal standard was investigated. However, 4-hydroxycyclophosphamide is not commercially available thus rendering it a less preferable internal standard. Furthermore, accuracy, within-day and between-day precisions were far within accepted limits, thus an internal standard was not considered essential. By adding 100 µl semicarbazide of pH 7.40 to 1 ml plasma with an approximate pH of 7.4 sufficient buffering capacity during LLE was accomplished. LLE of 1 ml plasma resulted in turbid reconstituted samples. However, by ultra-sonificating clear solutions were obtained with no chromatographic complications when 100 µl of the 150 µl reconstituted sample was injected.

Mass spectrometric identification of 4-hydroxyifosfamide-semicarbazide

The directly derivatized preparation and the eluted preparation of 4-hydroxyifosfamide-semicarbazide were both tested on the high-performance liquid chromatographic system as described in this article, before mass spectrometric analysis. No evidence of impurity could be detected with high-performance liquid chromatography, but the eluted preparation contained relatively high amounts of various salts originating from the mobile phase, which interfered with the mass spectrometric analysis. The mass-spectrogram of the preparative sample of 4-hydroxyifosfamide-semicarbazide is shown in figure 2.

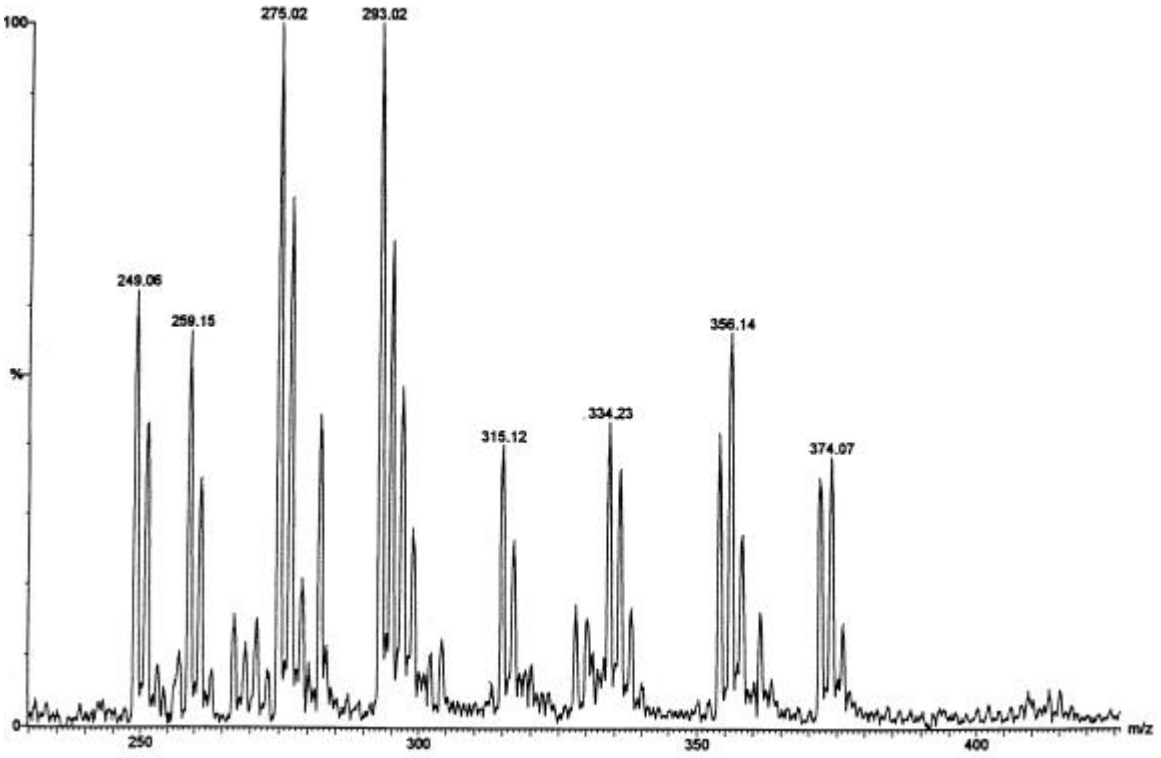

Figure 2. *Mass spectrogram of the derivative 4-hydroxyifosfamide-semicarbazone (M^+ 334) by means of electron spray electron impact mass spectrometry.*

At increasing ionization strengths more fragmentation was observed, but all spectra contained the parent peak with m/z of 334.1 (M^+) with its characteristic chlorine isotopes. The sodium adduct at m/z 356 (M^++Na) and the potassium adduct at m/z 374 (M^++K) confirmed the parent peak. Furthermore, a dimer (M_2^+) and trimer (M_3^+) with similar fragmentation patterns were observed albeit at 10 and 100 times lower intensities. The major fragmentation peaks were at m/z 80 ($NH_3^+CH_2CH_2Cl$), 249 (M^+-$CNNHCONH_2$), 259 (M^+-$NH_2NHCONH_2$), 275 (M^+-$NHCONH_2$). The peak at m/z 293 (M^+-$NHCONH_2$+H_2O) could be explained by addition of water (at the C-atom bonded to N-atom of semicarbazide) and subsequent fragmentation. The characteristic chlorine isotope fragmentation pattern suggested preservation of both chloroethyl-groups. Subsequently, the peak at m/z 315 (M^++Na-NHCN) was the sodium adduct of the rearranged fragment.

Specificity and selectivity

After normal sample pretreatment with semicarbazide and LLE, plasma samples spiked with ifosfamide, carboxyifosfamide, 2-dechloroethylifosfamide, 3-dechloroethylifosfamide, ifosforamide mustard, granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, topotecan and MESNA did not display any interference with the chromatography. Blank plasma and erythrocytes samples of six different individuals showed no interfering endogenous substances in the analysis of 4-hydroxyifosfamide-semicarbazide. Typical

chromatograms of blank erythrocyte and plasma samples and erythrocyte and plasma patient samples of 1.0 µg/ml are shown in figure 3.

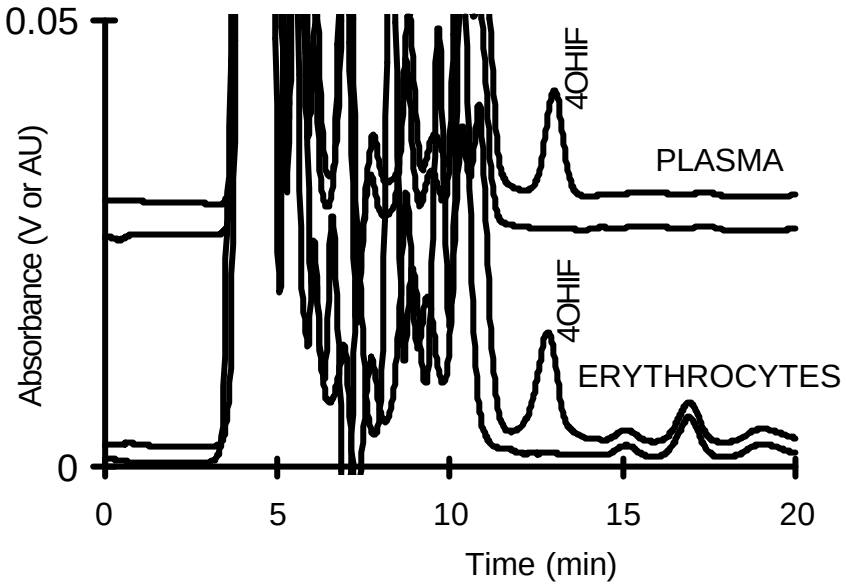

Figure 3. *Chromatograms of a 1-ml blank erythrocyte sample (lower curve of lower set of curves), a 1 ml patient erythrocyte sample containing 1.0 µg/ml 4-hydroxyifosfamide (upper curve of lower set of curves), a 1 ml blank plasma sample (lower curve of upper set of curves) and a 1 ml patient plasma sample containing 1.0 µg/ml 4-hydroxyifosfamide (upper curve of upper set of curves).*

However, the semicarbazide derivative of 4-ketoifosfamide did appear in the chromatogram (t_R 16.5 min.), well separated from 4-hydroxyifosfamide-semicarbazide (t_R 13.0 min.). Limited analysis of 4-ketoifosfamide revealed that the lower limit of quantification was approximately 5 µg/ml from a 1 ml plasma sample. Furthermore, 4-hydroxycyclophosphamide-semicarbazide (t_R 15.5 min.) can be detected, well separated from 4-hydroxyifosfamide-semicarbazide.

Limit of quantification and limit of detection

In three analytical runs the LLQ was determined in five-fold. The percent deviation from the nominal concentration (100 ng/ml) and the relative standard deviation were less than 18.1% and 7.5%, respectively. Thus, 100 ng/ml was defined to be the LLQ. The LOD in plasma was 10 ng/ml, with a signal-to-noise ratio of 3.

Validation: accuracy, precision, linearity and recovery

The results from the validation of the method in human plasma are listed in table 1. The use of the weight factor of $1/(conc.)^2$ resulted in a minimal sum of deviations from nominal concentrations. The method proved to be accurate (average accuracy at four different concentrations between 94.1 and 107.0% of the real concentrations) and precise (within-day precision ranged from 2.2 to 6.8% and between-day precision ranged from 2.6 to 7.2%). Correlation coefficients (r^2) of calibration curves were always higher than 0.995 as

determined by least squares analysis. Calibration curves proved to be linear in the range of 0.1-10 µg/ml with use of the *F*-test for lack of fit as an indicator of linearity of the regression model. Extraction recoveries from plasma were 96.0 ± 11.2 and 99.8 ± 3.7% for a 0.5 and 1.0 µg/ml 4-hydroxyifosfamide spiked sample (n=3), respectively. Similar, 4-hydroxyifosfamide was extracted from erythrocytes with 108.6 ± 9.5 and 118.8 ± 4.3% recovery, respectively. Since extraction recovery of erythrocytes were equal to that of plasma, a limited validation of the analysis in erythrocytes is sufficient.

Table 1. *Accuracy, within-day and between-day precision of the analysis of the 4-hydroxyifosfamide-semicarbazone derivative in human plasma, calculated using one-way ANOVA ($p<0.05$). Total number of different runs, total number of replicates per run for accuracy and total number of replicates per run for precision were respectively 3, 5 and 4.*

Concentrations	Accuracy (%)		Precision (%)		
(µg/ml)	Mean ± S.D.	95% C.I.	Mean ± S.D.	Within-day	Between-day
0.1	94.1±7.3	90.1-98.2	94.4±7.9	6.0	6.8
0.5	100.0±10.3	94.3-105.7	110.4±8.8	6.2	7.2
5.0	107.0±4.2	104.7-109.3	108.3±3.4	2.2	2.6
10.0	101.8±4.1	99.6-104.1	102.6±4.0	2.3	3.7

Abbreviations: S.D.= standard deviation, C.I.= confidence interval.

Optimization

Degradation during sample collection of underivatized 4-hydroxyifosfamide determined under various conditions is shown in table 2. With higher temperature and increasing time, breakdown is observed at both concentrations. Most relevant is the observation that at 4°C during the first 20 min. 4-hydroxyifosfamide is stable. The sample collection process in the clinic was always at 4°C and within 10 min., starting directly after collecting the blood until semicarbazide derivatization and storage. Optimal incubation during derivatization of 4-hydroxy-ifosfamide with semicarbazide is shown in table 3.

Table 2. *Effect of temperature and exposure time on degradation of 4-hydroxyifosfamide without semicarbazide protection in human plasma during sample collection.*

Temp.	Conc.	Degradation (% nominal conc.) ± C.V. (%)					N
	(µg/ml)	10 min.	20 min.	30 min.	60 min.	90 min.	
4°C	0.5	100.0±0.8	94.5±4.1	76.7±0.3	85.2±1.5	87.9±4.6	3
	5.0	100.0±2.7	94.4±1.7	93.8±3.0	93.0±0.5	83.0±0.6	3
25°C	0.5	98.0±1.5	94.3±1.6	75.7±3.6	57.9±2.0	56.5±2.4	3
	5.0	87.2±0.8	76.4±4.3	64.3±7.2	40.0±2.8	23.3±15.0	3
37°C	0.5	69.9±6.7	64.3±3.1	40.6±1.3	44.2±8.6	28.1±6.4	3
	5.0	67.0±4.6	39.8±4.9	18.1±13.4	9.9±6.5	7.8±14.9	3

Abbreviations: N=total number of replicates, C.V.=coefficient of variation.

Table 3. *Effect of temperature and incubation duration on derivatization of 4-hydroxyifosfamide with semicarbazone in human plasma during sample pretreatment.*

Temp.	Conc.	Recovery (%) ± C.V. (%)			N
	(µg/ml)	10 min.	20 min.	60 min.	
4°C	0.5	100.0±20.2	82.7±2.1	73.3±0.4	2
	5.0	77.7±6.3	100.0±11.4	89.1±1.2	2
25°C	0.5	65.0±0.4	51.0±10.7	40.4±5.5	2
	5.0	92.5±7.1	79.5±2.5	87.1±9.8	2
40°C	0.5	17.9±1.0	18.6±6.1	18.1±2.0	2
	5.0	52.3±4.1	46.1	47.2±1.2	2
60°C	0.5	22.1±3.4	15.9±9.0	19.6±0.7	2
	5.0	51.1±0.8	41.1±3.7	39.2±6.8	2

Abbreviations: N=total number of replicates, C.V.=coefficient of variation.

Belfayol et al. reported optimal incubation for the formation of 4-hydroxy-cyclophosphamide-semicarbazide at 60°C for 60 min.[10] Our investigations on the formation of 4-hydroxyifosfamide-semicarbazide show an optimal incubation at 4°C with minimal exposure. At 60°C for 60 min. a 60% reduction of 4-hydroxyifosfamide-semicarbazide can be observed. Although the reaction of 4-hydroxyifosfamide with semicarbazide might be maximized at higher temperatures, the still underivatized 4-hydroxyifosfamide is susceptible to breakdown in non-cooled circumstances. Therefore, the best method of derivatization appears to be a fast and cooled incubation with semicarbazide. This guarantees full albeit slow derivatization, but without unwanted degradation of 4-hydroxyifosfamide. Alteration of the amount of added semicarbazide (0.125-2 M) did not influence the intensity of the 4-hydroxyifosfamide-semicarbazide or the non-interfering endogenous peaks.

Stability during storage

The stability during storage of 4-hydroxyifosfamide-semicarbazide in biological matrix is represented in table 4. During the first month the degradation of 4-hydroxyifosfamide-

semicarbazide in biological matrix was less than 3% at -70°C and 10.6% at -20°C. Storage of pretreated 4-hydroxyifosfamide-semicarbazide samples (dry residues after LLE and solvent evaporation) was also stable for at least one month. By storing biological samples containing 4-hydroxyifosfamide-semicarbazide at most for one month at -20°C before analysis sample stability is not jeopardized.

Table 4. *Stability during storage of human plasma samples spiked with 4-hydroxyifosfamide-semicarbazone derivative.*

Storage	Conc.	Recovery (%) ± C.V. (%)					N
	(µg/ml)	1 hr.	24 hrs.	3 days	7 days	1 month	
60°C	0.5	100±1.7	52.6±1.4	n.d.	n.d.	n.d.	3
	5.0	100±0.5	47.4±0.7	n.d.	n.d.	n.d.	3
37°C	0.5	100±2.0	81.0±0.5	n.d.	n.d.	n.d.	3
	5.0	100±2.9	84.2±1.0	n.d.	n.d.	n.d.	3
25°C	0.5	100±1.3	91.9±3.9	74.4±1.4	73.0±4.8	n.d.	3
	5.0	100±2.4	89.7±0.7	79.5±3.1	77.6±1.5	n.d.	3
-20°C*	0.5	100.0±5.5	n.d.	105.5±4.1	101.8±5.5	100.6±3.1	3
	5.0	100.0±2.6	n.d.	104.9±0.8	93.2±2.3	89.4±1.7	3
-70°C*	0.5	100.0±6.6	n.d.	100.1±5.9	100.3±5.8	97.0±5.2	3
	5.0	100.0±2.2	n.d.	104.0±1.6	100.8±2.5	104.5±0.5	3

Abbreviations: N=total number of replicates, C.V.=coefficient of variation, *=including three freeze-thaw cycles, n.d.=not determined.

Analysis of patient samples

The applicability of the 4-hydroxyifosfamide assay for pharmacokinetic studies in patients receiving ifosfamide treatment was demonstrated, as depicted in figure 4. The assay allowed monitoring of plasma and erythrocytes levels of 4-hydroxyifosfamide. In addition, the erythrocytes can be used to investigate preferential transport routes of 4-hydroxyifosfamide throughout the body.[1] Since, 4-hydroxyifosfamide is the transport form directly prior in the cascade to the ultimate active metabolite ifosforamide mustard and the latter is unable to penetrate cell walls, it is the most relevant metabolite in blood to monitor ifosfamide pharmacokinetics and metabolism in relation to efficacy of ifosfamide treatment.

Conclusions

A validated assay for the quantitative determination of 4-hydroxyifosfamide in human plasma and erythrocytes has been described. The assay meets the current requirements as to the validation of a bioanalytical methodology and can be used for the pharmacokinetic studies with ifosfamide treated patients. It is our experience that the currently described high-performance liquid chromatographic assay can readily be used in a hospital laboratory environment for monitoring 4-hydroxyifosfamide concentrations.

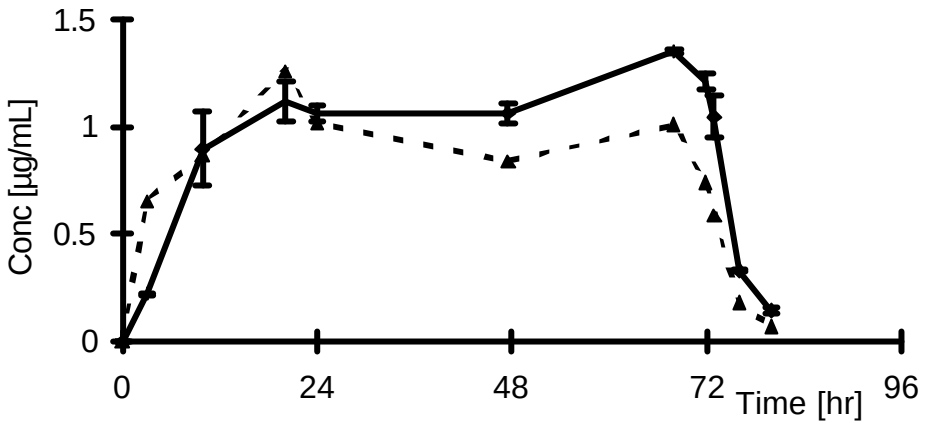

Figure 4. *4-Hydroxyifosfamide concentrations in plasma and erythrocytes (solid and dashed line, respectively) in a patient receiving a 72-hour intravenous infusion of 9 g/m^2 ifosfamide.*

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Chapter 2.3

Simultaneous determination of ifosfamide and its metabolite ifosforamide mustard in human plasma by high-performance liquid chromatography

Summary

Because ifosforamide mustard is the active alkylating metabolite of ifosfamide, it is of particular interest in the pharmacokinetic analysis of patients undergoing ifosfamide treatment. This paper presents an assay for the simultaneous determination of ifosforamide mustard and ifosfamide after derivatization with diethyldithiocarbamate (DDTC), subsequent liquid-liquid extraction of the plasma with acetonitrile and using reversed phase high-performance liquid chromatography with ultra-violet detection at 276 nm. Structural confirmation of the analytes was accomplished using mass spectrometry (MS). Reaction conditions such as incubation duration, temperature and concentration of derivatization agent were investigated; 30 min at 70°C with 100 mg/ml DDTC was optimal. The presented analytical method proved to be accurate, precise and linear for ifosforamide mustard and ifosfamide concentrations, ranging from 0.100-50.0 and 0.100-100 µg/ml, respectively, and with lower limits of quantification of 0.100 µg/ml for both analytes. A typical patient pharmacokinetic profile is presented to demonstrate the applicability of the assay in clinical samples. The analytical method could be employed in high-throughput clinical analysis of ifosforamide mustard and ifosfamide patient samples.

Introduction

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide

(Holoxan®, Ifex®) is commonly used for the treatment of various solid tumours, soft tissue sarcomas and haematological malignancies in adults and children. Ifosfamide is a prodrug, which needs activation via the cytochrome P450 enzymatic system in the liver to exert its alkylating activity.[1] First, ifosfamide is hydroxylated to 4-hydroxyifosfamide as represented in figure 1.

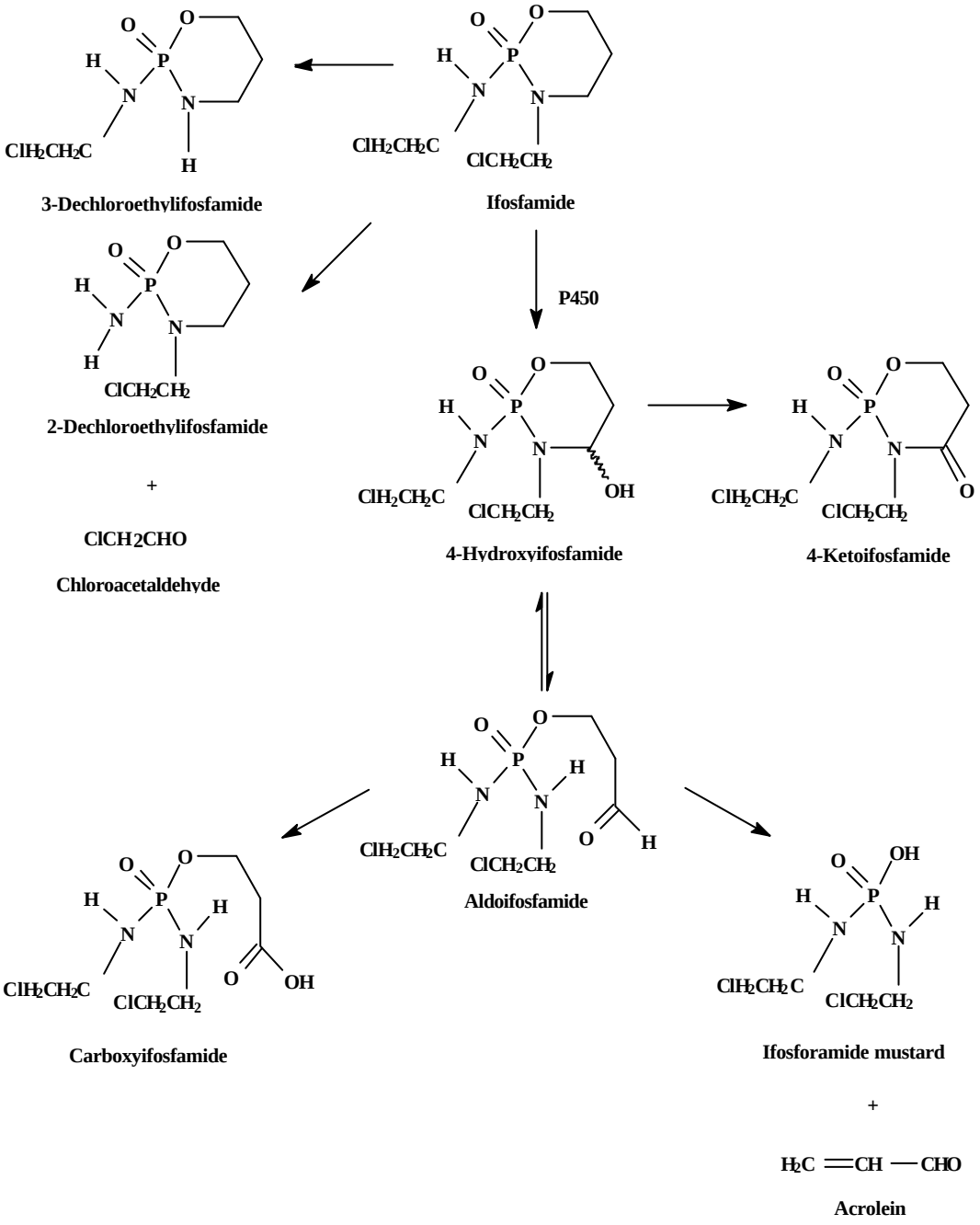

Figure 1. *Metabolism of ifosfamide.*

4-Hydroxyifosfamide exists in equilibrium with its tautomeric form aldoifosfamide. Aldoifosfamide can be converted into the final alkylating agent ifosforamide mustard with concurrent acrolein formation. Besides activation de-activation occurs, yielding 2-dechloroethylifosfamide and 3-dechloroethylifosfamide with equimolar release of neurotoxic

chloroacetaldehyde. In the (tumour)cell ifosforamide mustard reacts with DNA causing interstrand cross-links, leading to cell death.[2] Ifosfamide is known to increase its own metabolism (autoinduction).[3]

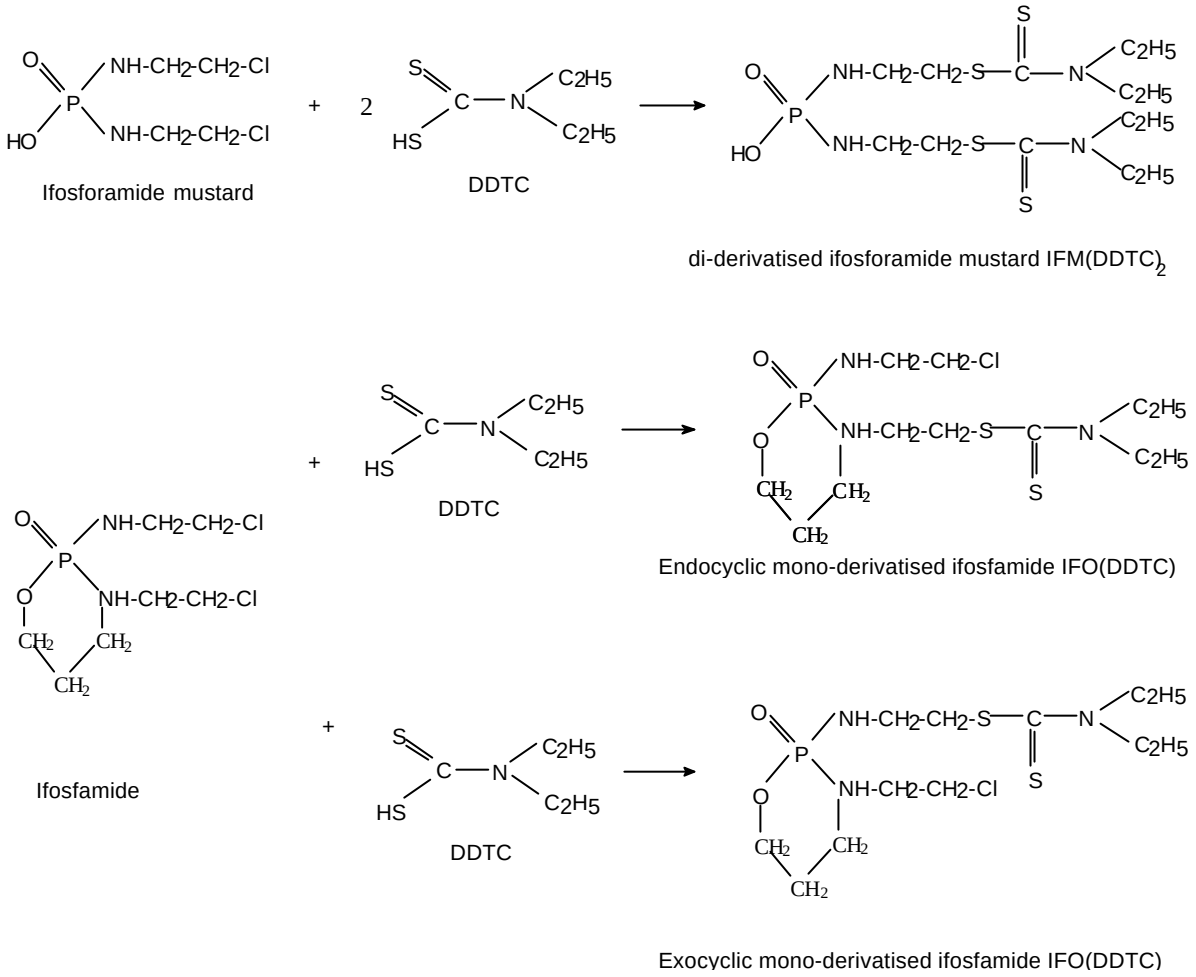

Figure 2. *Derivatization of ifosforamide mustard and ifosfamide with diethyldithiocarbamate (DDTC).*

Recently, an assay was reported for ifosforamide mustard.[4] This method consisted of direct derivatization of ifosforamide mustard in plasma with diethyldithiocarbamate (DDTC) and subsequent solid phase extraction (SPE) of the resulting derivative. The samples were stabilized by adding semicarbazide and sodium chloride, which prevent auto-catalytic breakdown of 4-hydroxyifosfamide to ifosforamide mustard and further ifosforamide mustard breakdown, respectively. During large scale implementation of this method various problems were encountered. These were mainly clogging of the SPE columns, low analytical column half-life, and late co-eluting peaks, preventing rapid use in clinical investigations with high sample throughput. A faster more reproducible method has now been developed for ifosforamide mustard, which also permitted simultaneous determination of ifosfamide. No easy applicable assay for both ifosforamide mustard and ifosfamide by high-performance liquid chromatography has been described thus far. Derivatization of ifosforamide mustard (IFM) and ifosfamide (IFO) with DDTC leads to IFM(DDTC)$_2$ and IFO(DDTC) as depicted in figure 2. Structural confirmation of the analytes and assay validation were performed for both

ifosforamide mustard and ifosfamide.

Materials and Methods

Equipment

The high-performance liquid chromatographic equipment consisted of an integrated system with a model SCM1000 in-line degassing unit, a model P1000 pump, a model AS3000 automated injector with a 100-µl loop, a model UV150 ultra-violet (UV) detector operated at 276 nm and a DataJet integrator (Thermo Separation Products, Breda, The Netherlands). Data were analysed using PC1000 software (Thermo Separation Products, Breda, The Netherlands) using a Dell optiplex Gs Pentium personal computer. A 250 x 40 mm I.D. RP8 5 µm particle size Prodigy 5 C8 column with a Security Guard C8 pre-column (Phenomenex, Torrance, CA, USA) was operated at ambient temperature. The analytical column was washed monthly with 1-propanol in order to maintain high separation efficiency. The mobile phase consisted of acetonitrile (AcN):water (32:68 v/v) containing 25 mM K_2HPO_4 buffer pH 8.0. For identification of the derivatives a mass spectrometer (MS) was used operating with direct continuous split-injection (Sciex, Thornhill, ON, Canada). Electrospray ionization (1500 V) was achieved using a TurboIon™ sample inlet. Ions were created at atmospheric pressure and were transferred to a model API 365 triple quadrupole mass spectrometer (LC/ESI-MS/MS) with a dwell-time of 0.1-1 msec. The channel electron multiplier was set at 1900 V. For optimising the wavelength of detection, the UV-absorption spectrum was recorded on-line using a Waters model 996 Photodiode-Array (PDA) detector (Waters Chromatography, Milford, Ma, USA).

Chemicals

Ifosforamide mustard (D-18847, Lot 034.5333) and all other ifosfamide and cyclophosphamide metabolites were a kind gift of Dr J. Pohl, ASTA Medica AG (Frankfurt, Germany). Sodium diethyldithiocarbamate was obtained from Sigma (St. Louis, Mo, USA). AcN and methanol (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). K_2HPO_4, sodium chloride, hydrochloric acid and sodium hydroxide (suprapure grade) were obtained from Merck (Darmstadt, Germany). Semicarbazide hydrochloride (p.a. grade) was purchased from Acros (Geel, Belgium). Distilled water was used throughout.

Preparation of standards

Blank, drug-free plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Plasma was centrifuged at 1,000 g for 5 min and the pellet was discarded. To the supernatant a solution containing 2 M semicarbazide, 1 M sodium chloride pH 7.40 (SCZ*) was added, yielding a mixture of plasma:SCZ* 10:1 v/v (plasma*). After storage of at least one hour at -70°C the plasma* was thawed and centrifuged again at 1,000 g for 5 min. The supernatant was used as blank plasma in the assay. Ifosforamide mustard was dissolved as a 1 mg/ml solution in a phosphate buffer (1 M sodium chloride, 0.1 M K_2HPO_4, pH=8.00)(PPB) immediately prior to preparation of the

standards. Ifosfamide was also dissolved as a 1 mg/ml solution in PPB. 350 µl Ifosforamide mustard and ifosfamide solutions in PPB were added to 500 µl plasma* in a 2.0 ml Eppendorf cup, resulting in combined calibration curves of 0, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0 and 50.00 µg/ml ifosforamide mustard and 0, 0.100, 0.500, 1.00, 5.00, 10.0, 25.0, 50.0 and 100 µg/ml ifosfamide. Quality control samples were prepared similarly but from different stock solutions and separate weighting of the standards at 0.100, 0.250, 5.00 and 50.0 µg/ml ifosforamide mustard and 0.100, 0.500, 50.0 and 100 µg/ml ifosfamide.

Sample pretreatment

The derivatising agent was added as a 50-µl volume of 100 mg/ml DDTC in PPB to 500 µl plasma* and 350 µl PPB. After whirl-mixing for 15 sec the samples were placed for 30 min at 70°C in a thermostatically controlled waterbath. After incubation the samples were placed on ice water in order to stop the derivatization reaction. Liquid-liquid extraction was performed by adding 1,000 µl AcN. After whirl-mixing thoroughly three times for 1 min and subsequent centrifugation at 3,000 g for 10 min at 4°C, a 700-µl volume of the AcN fraction was transferred to a clean 1.5 ml Eppendorf cup and evaporated to dryness under a gentile stream of nitrogen at 40°C. The samples were reconstituted by adding 150 µl mobile phase. After whirl-mixing for 1 min and subsequent centrifugation at 3,000 g for 5 min at ambient temperature, the supernatants of the samples were transferred to 200 µl inserts in vials. The injected volume was 50 µl.

Patient sample collection and pretreatment

Whole blood samples were collected and immediately placed on ice water, centrifuged at 1,000 g for 5 min at 4°C and 1 ml plasma was transferred to a 2-ml Eppendorf cup containing 100 µl SCZ*, yielding plasma*. After thorough vortex mixing the samples were stored at -70°C. The entire sample handling was always performed within 10 min. After thawing, a 500-µl volume of patient plasma* was prepared in duplicate, identical to standard sample pretreatment.

Identification

Identification of derivatized ifosforamide mustard was achieved by derivatising an 850 µl volume of 5 mg/ml ifosforamide mustard in PPB with 50 µl 500 mg/ml DDTC in water and incubation and extraction with AcN as described for the plasma samples. After evaporation of the organic extract, the sample was reconstituted in 100 µl mobile phase and injected in the high-performance liquid chromatographic system. The analyte was isolated by collecting the fraction containing the ifosforamide mustard-derivative. The eluate was again extracted with 2:1 (v/v) AcN after addition of 1 g sodium chloride. After whirl-mixing and centrifugation the AcN-layer was transferred and evaporated as described above. Since, the residue could be contaminated by sodium chloride dissolved in co-extracted water in the AcN-layer, the dry residue was again extracted with 1 ml AcN. After transfer of the AcN-layer and subsequent evaporation the clean residue was stored at -70°C pending identification by MS. Derivatization

of ifosfamide yields two products as represented in figure 2, with two corresponding peaks in the high-performance liquid chromatographic chromatogram. The ifosfamide-derivatives were isolated similarly by collecting both peaks separately. Partial re-injection of the final AcN-layer on the high-performance liquid chromatographic system confirmed the purity of the isolated ifosforamide mustard and ifosfamide-derivatives. The isolated derivatives were reconstituted in 80% methanol solution and analysed by MS for identification.

Optimization of derivatization

Derivatization conditions were changed to find the optimal incubation temperature, duration and DDTC concentration. Variations in conditions were tested with 0.500 and 5.00 µg/ml ifosforamide mustard spiked plasma in duplicate. Derivatization temperature was investigated at ambient temperature (20°C), 40°C and 70°C for 5, 10, 30 and 60 min and the reaction duration at 70°C for 0, 5, 10, 20, 30, 60 and 120 min. To find the optimal derivatization reagent concentration tests were performed at 70°C for 30 min with 0, 0.100, 0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 mg/ml DDTC.

Specificity and selectivity

Potential interference from endogenous compounds was investigated by analysing six different blank plasma samples. The following compounds were investigated for interference with the analytical method: 2-dechloroethylifosfamide, 3-dechloroethyl-ifosfamide, didechloroethylifosfamide, 4-ketoifosfamide, carboxyifosfamide, 4-hydroxy-ifosfamide with and 4-hydroxyifosfamide without semicarbazide stabilization. Possible co-medication was also tested for interference. Tested substances were topotecan, paclitaxel, ketoconazole, granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, caffeine, furosemide and sodium 2-mercaptoethane sulphonate (MESNA). All compounds were tested at a concentration of 20 µg/ml.

Limit of quantification

The lower limits of quantification (LLQ) of ifosforamide mustard and ifosfamide were investigated in plasma samples from three different donors by a five-fold determination in three analytical runs. For the concentration to be accepted as the LLQ, the percentage deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) were to be less than 20%. The upper limits of quantification (ULQ) of ifosforamide mustard and ifosfamide were defined as 50.0 and 100 µg/ml, respectively, based upon the expected clinical concentration range.

Validation: accuracy, precision and linearity

Accuracy, between-day and within-day precisions of the method were determined by assaying five replicate quality control samples in plasma at four different ifosforamide mustard and ifosfamide concentrations (0.100, 0.250, 5.00 and 50.0 µg/ml and 0.100, 0.500, 50.0 and 100 µg/ml, respectively) in three different analytical runs. Accuracy was measured

as a percentage of the nominal concentration. The within-day and between-day precisions were obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as grouping variable. For the construction of each calibration curve eight spiked plasma samples were analysed in duplicate. After optimization of their weighting factors, linearity of the plasma calibration curves was tested with the *F*-test for lack of fit.

Extraction recovery

Recovery of extraction of the ifosforamide mustard-derivative was determined by dissolving the isolated ifosforamide mustard-derivative in PPB, resulting in theoretical 1.00 and 10.0 µg/ml solutions which were diluted further. Full recovery (without sample extraction) was achieved by addition of AcN to the derivative solution in PPB (68:32 v/v), producing a solution identical to the mobile phase, which was injected directly into the high-performance liquid chromatographic system. Spiked plasma samples were extracted, transferred, evaporated and subsequently reconstituted in mobile phase and injected on the high-performance liquid chromatographic system as described above. Furthermore, 25 mM K_2HPO_4 pH 8.00 was also added to the AcN-layer after extraction (68:32 v/v) of additional samples, producing a solution identical to the mobile phase. Recoveries were calculated by comparing the directly injected ifosforamide mustard-derivative to the extracted samples with and without the evaporation-step.

Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *p*-values were 0.05 or less.

Results and discussion

Chromatography and detection

The chromatographic method enabled sufficient separation of the analytes. No ion-exchange modifier was used since it did not prove to be essential and better column endurance could be obtained without it. Figure 3 represents typical chromatograms of a blank sample, a patient sample of 3.20 and 14.9 µg/ml ifosforamide mustard and ifosfamide, respectively, and a spiked sample of 5.00 and 25.0 µg/ml ifosforamide mustard and ifosfamide, respectively.

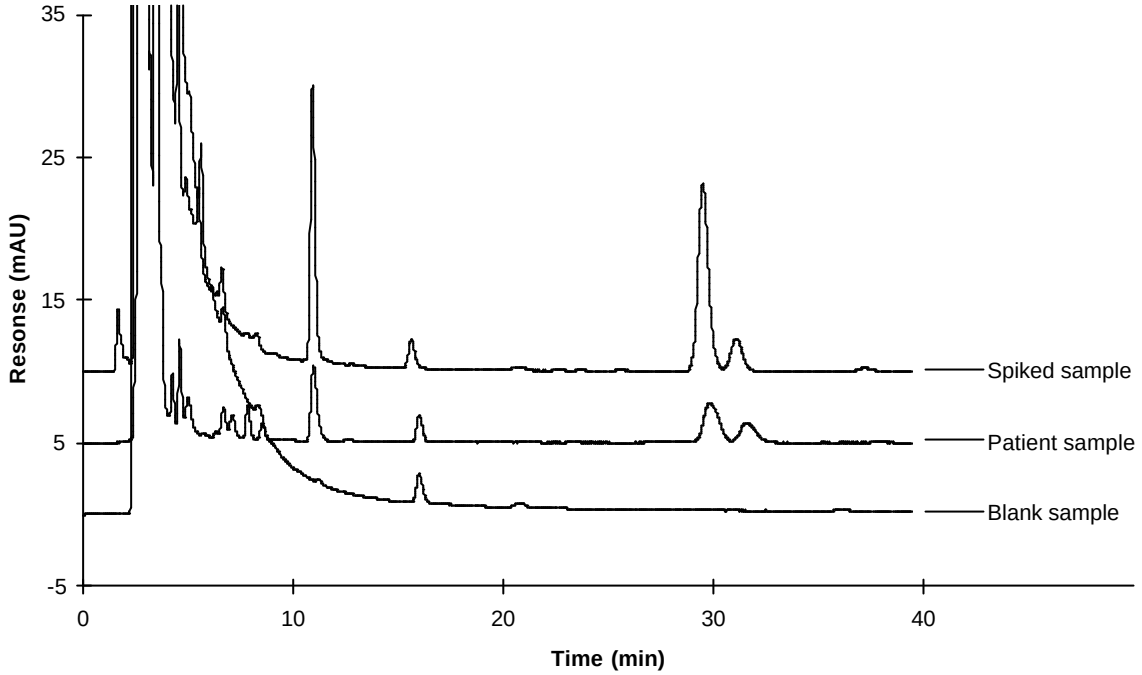

Figure 3. *Typical chromatograms of a blank (bottom), a patient (middle) and a spiked (top) plasma sample with 0, 3.20 and 5.00 µg/ml ifosforamide mustard (11.0 min) and 0, 14.9 and 25.0 µg/ml ifosfamide (29.8 and 31.5 min), respectively.*

The patient sample was taken at the end of a 1-hour 1.2 g/m² ifosfamide intravenous (i.v.) infusion. Noteworthy are the large injection peak consisting of non-retained DDTC and (derivatized) matrix components, the IFM$(DDTC)_2$ peak at 11.0 min and the IFO(DDTC) peaks at 29.8 and 31.5 min, respectively. Since the ratio of the exo- and endocyclic ifosfamide-derivative peaks was always equal to 5:1 and it can be expected that the molar absorptivities of the compounds are identical, the sum of both peaks was used in the quantification of ifosfamide.

Sample pretreatment

Besides stabilization of underivatized ifosforamide mustard,[4] the addition of sodium chloride in PPB to the plasma sample also prevented mixing of the aqueous and organic-phases during the liquid-liquid extraction enabling easy transfer of the organic-layer and preventing undesired co-extraction of water-soluble plasma components. SCZ* addition prevented autocatalytic degradation of 4-hydroxyifosfamide. After a freeze-thaw cycle and subsequent centrifugation, addition of SCZ* to blank or patient plasma resulted in a clear plasma layer and a pellet. If the plasma was not subjected to this procedure no reproducible separation of the aqueous and organic-layer could be obtained. The pellet possibly contained plasma components that otherwise prevent a clear separation of the phases.

Identification

The peak of the ifosforamide mustard-derivative was identified as double derivatized IFM$(DDTC)_2$, as depicted in figure 4. The molecular weight of IFM$(DDTC)_2$ is 446.1, as depicted in figure 2. Consequently, the parent-peak (MH^+) can be observed at a mass-to-charge ratio (m/z) of 447. The sodium and potassium-adducts were observed at m/z 469 and 485, respectively. A dimer (M^+_2) was observed at m/z 892 with its typical sodium-adduct at m/z 915. The fragment at m/z 116 and m/z 176 may be explained by $CSN(C_2H_5)_2$ and $C_2H_5SCSN(C_2H_5)_2$ fragments, respectively. The two ifosfamide peaks were identified as isomeric monomers of IFO(DDTC), as depicted in figure 2. The DDTC could react with either the chloroethylgroup attached to the exo- or endocyclic nitrogen atom. The molecular weight of IFO(DDTC) is 373.1. Figures 5 and 6 demonstrate similar MS patterns for both isomers. Although differences in relative intensity between fragments were observed, no unique fragments could be distinguished between the two isomers. The MH^+ was observed at m/z 374 with its typical Cl atom isotope peaks. The sodium and potassium-adducts were observed at m/z 396 and 411. A dimer (M^+_2) was observed at m/z 746 with its sodium and potassium-adducts at m/z 769 and 785. Since none of the theoretical possible fragments of either the exo- or endocyclic derivatized ifosfamide were observed uniquely with MS, further identification was performed by refragmentation of the parent-peak (MS^2). MS^2 of peak 1 yielded fragments at m/z 92 and 120. These fragments may be explained by $CH_2NHC_2H_4Cl$ and $C_3H_6NHC_2H_4Cl$, respectively.

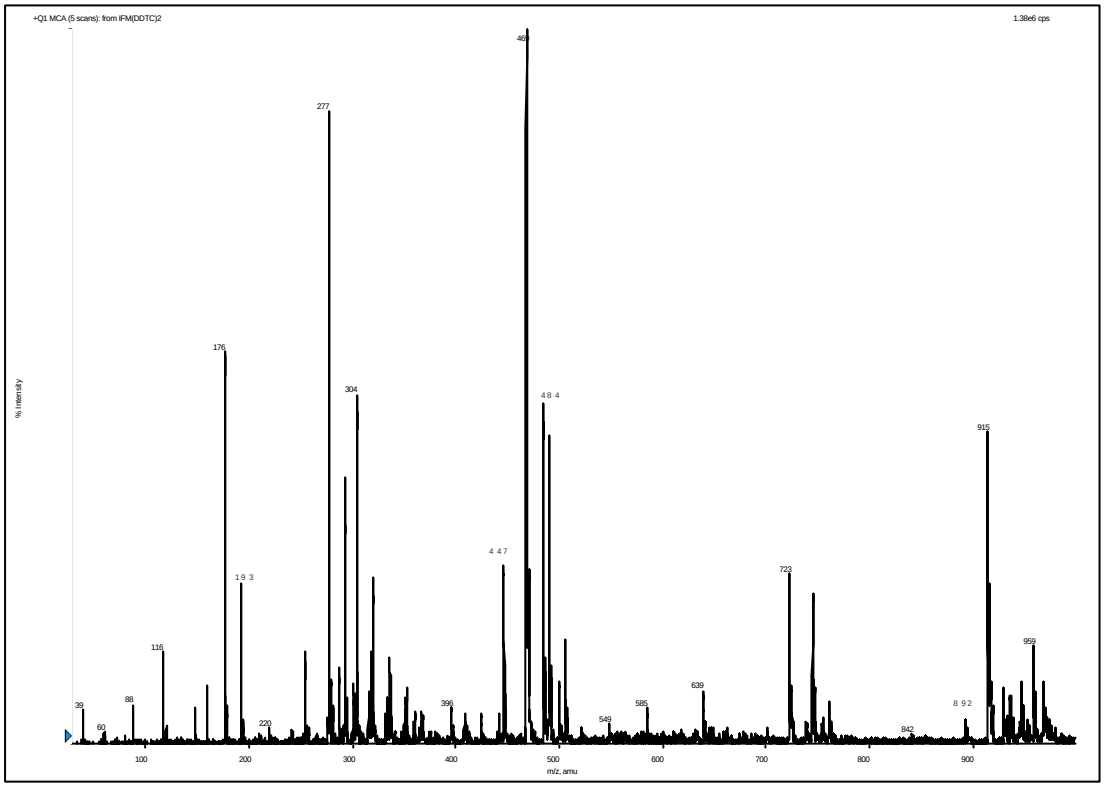

Figure 4. *Mass-spectrum of ifosforamide mustard di-derivative (IFM(DDTC)$_2$). IFM(DDTC)$_2$ was isolated at 11.0 min.*

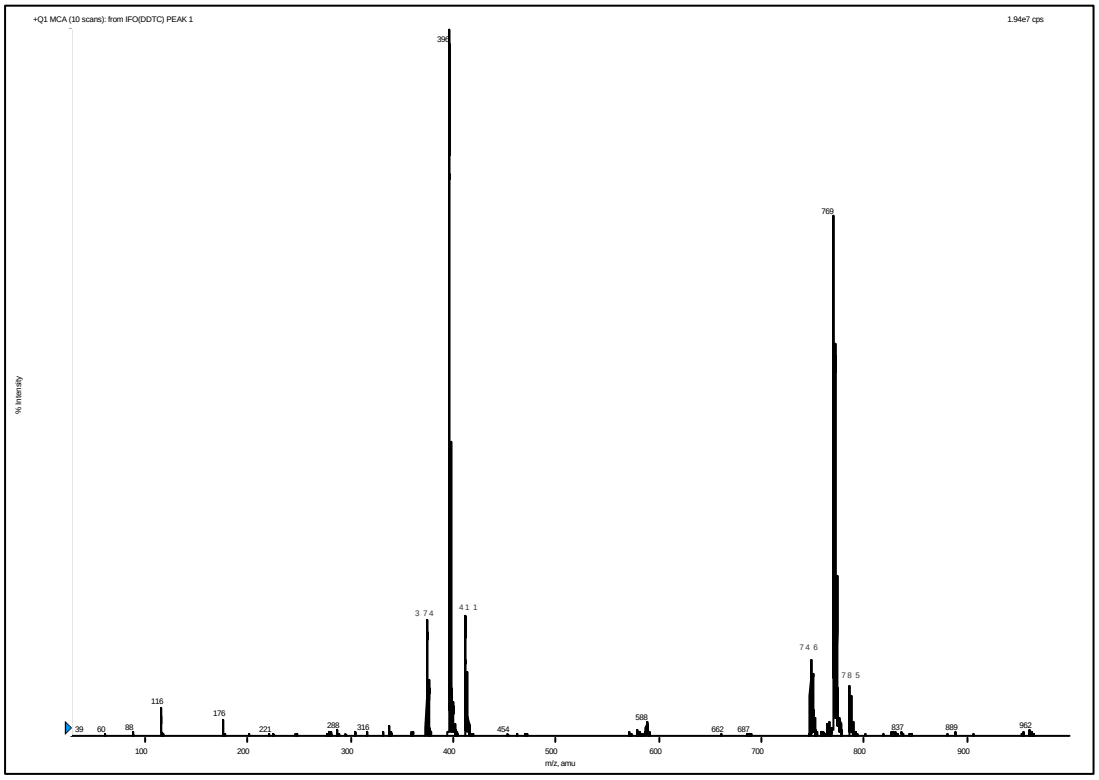

Figure 5. *Mass-spectrum of the exocyclic ifosfamide mono-derivative (IFO(DDTC)) isolated at 29.8 min.*

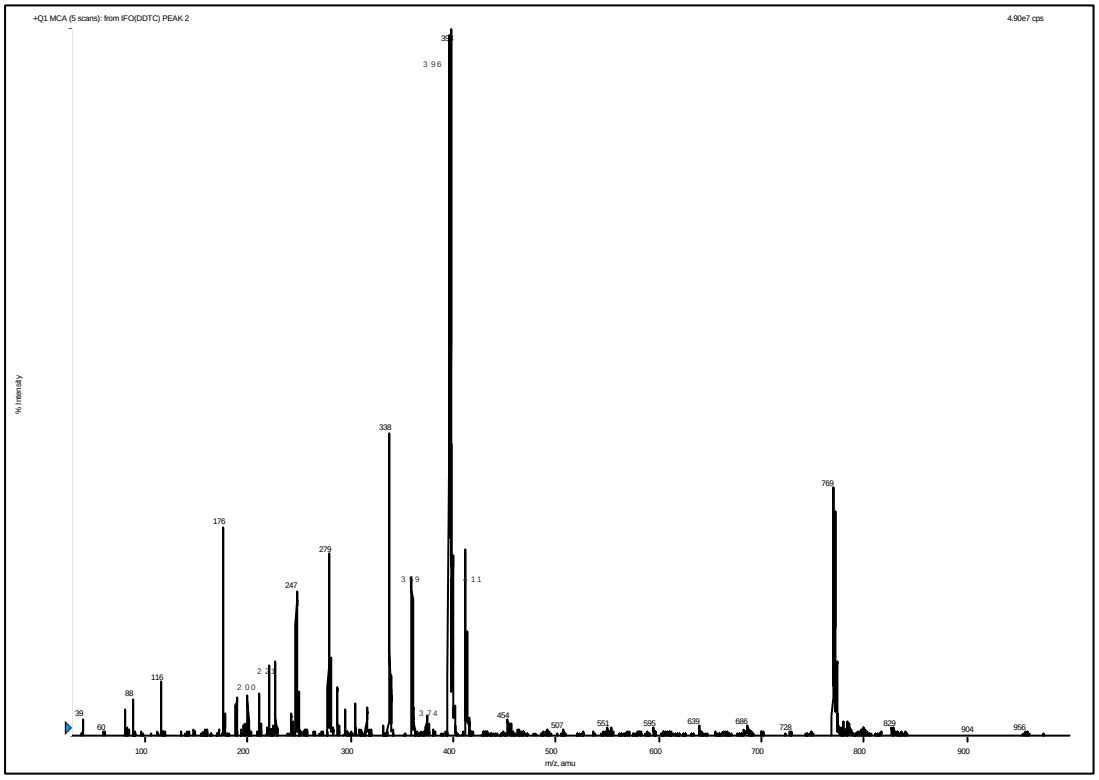

Figure 6. *Mass-spectrum of the endocyclic ifosfamide mono-derivative (IFO(DDTC)) isolated at 31.5 min.*

The exocyclic derivative can yield these fragments, the endocyclic derivative cannot. Therefore, it was concluded that ifosfamide peak 1 was the exocyclic derivatized ifosfamide and ifosfamide peak 2 was the endocyclic derivatized ifosfamide. The UV-absorption spectrum is shown in figure 7. A maximum is observed at 276 nm.

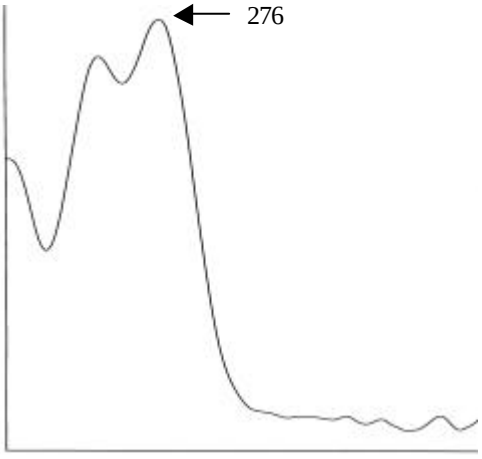

Figure 7. *Ultra-violet absorption spectrum of ifosforamide mustard di-derivative.*

Specificity and selectivity

After sample pretreatment, plasma samples spiked with 2-dechloroethylifosfamide, didechloroethylifosfamide, carboxyifosfamide, topotecan, paclitaxel, acetaminophen, caffeine, furosemide and MESNA did not display any interference with the method. Non-interfering peaks were detected for 3-dechloroethylifosfamide (9.0 min), 4-ketoifosfamide (19.8 min), ketoconazole (83.5 min), granisetron (6.0 min), dexamethasone (12.5 min), oxazepam (14.5, 21.3 and 26.4 min), temazepam (22.8 min), compared to ifosforamide mustard (11.0 min). Stabilized 4-hydroxyifosfamide (with semicarbazide) resulted in a peak at 11.4 min. Unstabilized 4-hydroxyifosfamide was spontaneously converted to ifosforamide mustard (11.0 min). Blank plasma samples of six different individuals showed no interfering endogenous substances in the analysis. Addition of semicarbazide did not interfere with the chromatography.

Optimization of derivatization

The derivatization temperature did not demonstrate an optimum, but the variability with 0.500 and 5.00 µg/ml ifosforamide mustard at 70°C was less than at 20°C and 40°C. Therefore, 70°C was selected as derivatization temperature. Optimization of derivatization duration scaled to 100% is presented in figure 8.

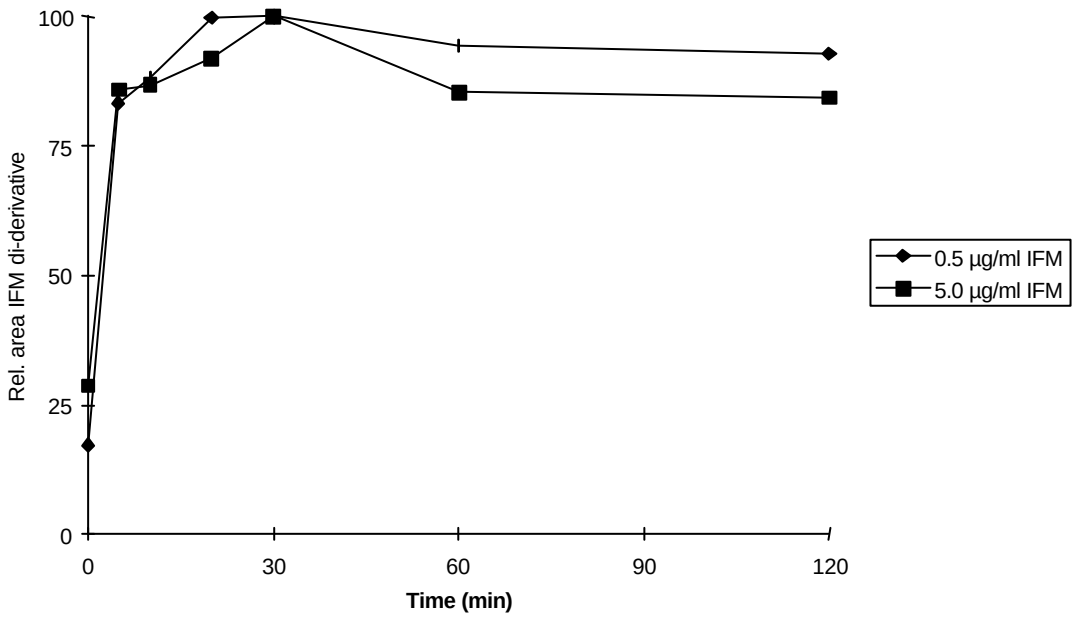

Figure 8. *Effect of incubation duration on derivatization of ifosforamide mustard (IFM) with diethyldithiocarbamate.*

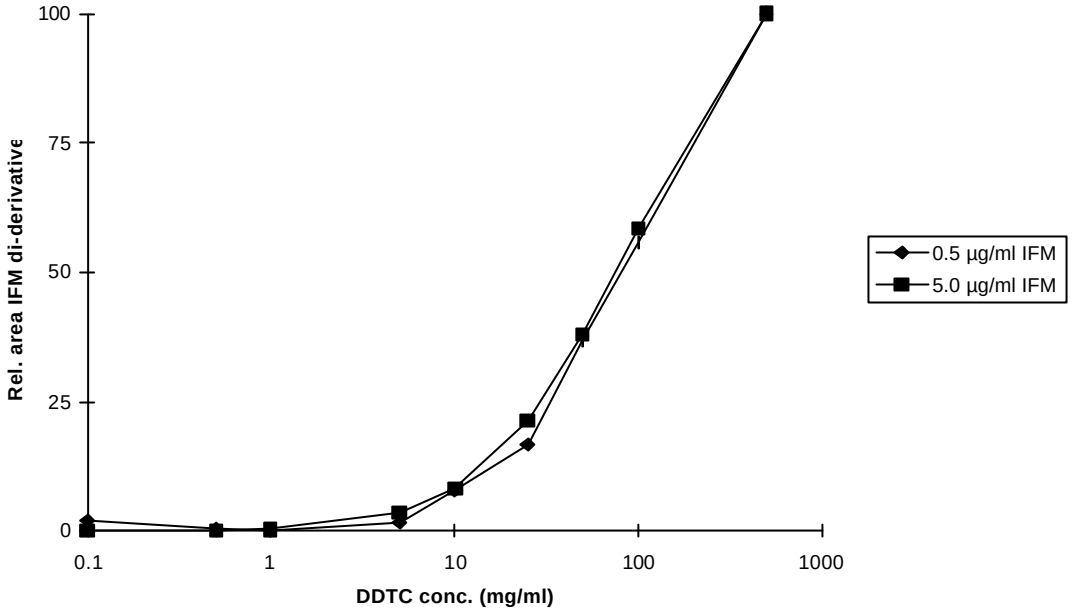

Figure 9. *Effect of diethyldithiocarbamate (DDTC) concentration on derivatization of ifosforamide mustard (IFM).*

The optimum derivatization duration was set at 30 min. The effect of DDTC concentration on the derivatization of ifosforamide mustard is presented in figure 9. Between 0 and 5.00 mg/ml no ifosforamide mustard-derivative could be detected. Between 25.0 and 500 mg/ml DDTC a log-linear relationship between derivative response and DDTC concentration is observed. Although no optimum was reached, chromatographic separation between the unretained DDTC peak and the ifosforamide mustard-derivative deteriorated at reagent concentrations of 500 mg/ml. Therefore, 100 mg/ml DDTC was chosen as the most optimal concentration for derivatization.

Limit of quantification

In three analytical runs the LLQs of ifosforamide mustard and ifosfamide were determined in five-fold. The mean percentage deviation from the nominal concentration at 0.100 µg/ml ifosforamide mustard and ifosfamide were 10.2 and 1.9% with a relative standard deviation of 17.2 and 11.0%, respectively. Therefore, both LLQs were determined to be 0.100 µg/ml.

Validation: accuracy, precision and linearity

The results from the validation of the method in human plasma are listed in table 1. The use of the weighting factor of $1/(conc.)^2$ resulted in a minimal sum of squares of residuals from the nominal concentrations. The method proved to be accurate for both analytes (average accuracy at four different concentrations between 89.5 and 110.0% of the nominal concentrations) and precise (within-day precision ranged from 2.8 to 15.8% and between-day precision ranged from 2.0 to 10.0%). Correlation coefficients (r^2) of calibration curves were always higher than 0.995 as determined by least sum of squares analysis. Calibration curves of ifosforamide mustard and ifosfamide proved to be linear in the range of 0.100-50.0 and

0.100-100 µg/ml, respectively, without lack of fit. Furthermore, no systematic proportional or additive errors were observed.

Table 1. *Accuracy, within-day and between-day precisions of ifosforamide mustard (IFM) and ifosfamide (IFO) analysis in human plasma.*

Analyte	Concentrations	Accuracy (%)		Precision (%)	
	(µg/ml)	Mean ± S.D.	95% C.I.	Within-day	Between-day
IFM	0.100	110.0±17.0	100.6-119.7	15.8	N.A.
IFM	0.250	107.2±15.2	98.7-115.4	11.3	10.0
IFM	5.00	106.6±8.0	102.1-111.0	4.5	7.2
IFM	50.0	101.4±8.3	96.8-106.0	2.8	9.2
IFO	0.100	101.9±11.0	95.8-108.0	11.2	N.A.
IFO	0.500	94.6±5.1	91.8-97.4	5.1	2.0
IFO	50.0	90.6±5.3	87.6-93.5	4.2	4.9
IFO	100	89.5±3.6	87.5-91.5	4.0	N.A.

Abbreviations: S.D.= standard deviation, C.I.= confidence interval, N.A.= not applicable, between-day variation did not exceed within-day variation.

Extraction recovery

Extraction recoveries (mean ± coefficient of variation) of the ifosforamide mustard-derivative from plasma with the evaporation-step were 62.2 ± 6.7 and 62.4 ± 13.4% for 1.00 and 10.0 µg/ml ifosforamide mustard (n=3), respectively. Extraction recoveries of the ifosforamide mustard-derivative from plasma without evaporation-step were 62.9 ± 8.4 and 59.8 ± 3.3% for 1.00 and 10.0 µg/ml ifosforamide mustard spiked sample (n=3), respectively. It is obvious that there is no significant extraction-loss of ifosforamide mustard due to the evaporation-step.

Analysis of patient samples

Figure 10 represents a typical pharmacokinetic profile of a patient receiving 3 g/m² ifosfamide i.v. in three hours on two consecutive days. Ifosfamide and ifosforamide mustard concentrations accumulated over time, but were also eliminated faster on the second day. This resulted in similar maximum ifosfamide concentrations on day 1 and 2. But ifosforamide mustard concentrations were substantially higher on day 2, because more ifosfamide was metabolized to ifosforamide mustard on day 2. This phenomenon can be explained by the autoinduction of ifosfamide and its effect on the metabolism.

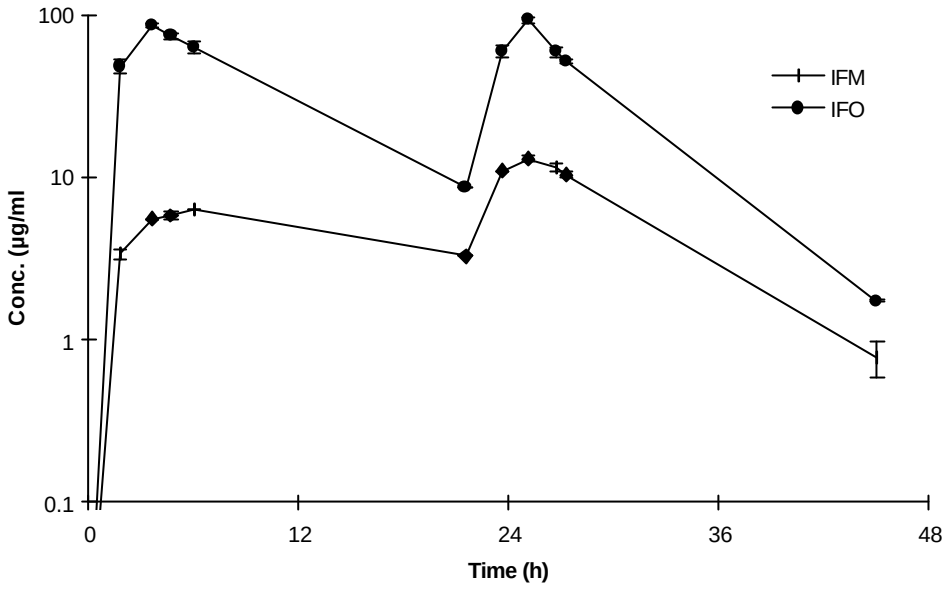

Figure 10. *Ifosforamide mustard (IFM, lower curve) and ifosfamide (IFO, upper curve) concentrations in plasma in a patient receiving 3 g/m² ifosfamide i.v. in three hours on two consecutive days.*

Conclusions

An analytical method for the simultaneous determination of ifosforamide mustard and ifosfamide in human plasma was described. This technique employed derivatization with DDTC followed by deproteinization with AcN. Quantification was achieved by reversed phase high-performance liquid chromatographic with UV-detection. Identification of the derivatized analytes was accomplished using MS and LC-PDA. Incubation settings were optimized. It is our experience that the presented assay can be readily used in a hospital laboratory environment for simultaneous monitoring of ifosforamide mustard and ifosfamide concentrations in patients.

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Chapter 2.4

Distribution of ifosfamide and metabolites between plasma and erythrocytes

Summary

The distribution of ifosfamide and its metabolites 2-dechloroethylifosfamide, 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard between plasma and erythrocytes was examined in vitro and in vivo. In vitro distribution was investigated by incubating whole blood with various concentrations of ifosfamide and its metabolites followed by separation of plasma and erythrocyte layers and subsequent analysis. In vivo distribution of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide was determined in seven patients receiving a 72-hour intravenous continuous infusion of 9 g/m² ifosfamide. In vitro distribution equilibrium between erythrocytes and plasma was obtained quickly after drug addition. Mean (±sem) in vitro and in vivo erythrocyte (e)-plasma (p) partition coefficients ($P_{e/p}$) were 0.75±0.01 and 0.81±0.03, 0.62±0.09 and 0.73±0.05, 0.76±0.10 and 0.93±0.05 and 1.38±0.04 and 0.98±0.09 for ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide respectively. These ratios were independent of concentration and unaltered with time. The ratios of the area under the erythrocyte and plasma concentration-time curves ($AUC_{e/p}$) obtained in patients were 0.96±0.03, 0.87±0.07, 0.98±0.06 and 1.34±0.39, respectively. A time- and concentration dependent distribution-equilibrium phenomenon was observed during incubation with ifosforamide mustard. It was hypothesized that the more hydrophilic ifosforamide mustard is less capable of penetrating the erythrocyte compared to the other analytes. Complete spontaneous in vitro conversion of 4-hydroxyifosfamide to ifosforamide mustard was observed. The equivalency in red blood cell partitioning of ifosfamide and its metabolites under in vitro and in vivo conditions was corroborated by the $AUC_{e/p}$s, because similar ratios were observed. It is concluded that ifosfamide and metabolites rapidly reach distribution equilibrium between erythrocytes and plasma; the process is slower for ifosforamide mustard. Drug distribution to the erythrocyte fraction ranged from about 38% for 2-dechloroethylifosfamide to 58% for 4-hydroxyifosfamide

and was stable over a wide range of clinically relevant concentrations. A strong parallelism in the erythrocyte and plasma concentration profiles was observed for all compounds. Thus, pharmacokinetic assessment using only plasma sampling yields direct and accurate insights into the whole blood kinetics of ifosfamide and metabolites and may be used for pharmacokinetic-pharmacodynamic studies.

Introduction

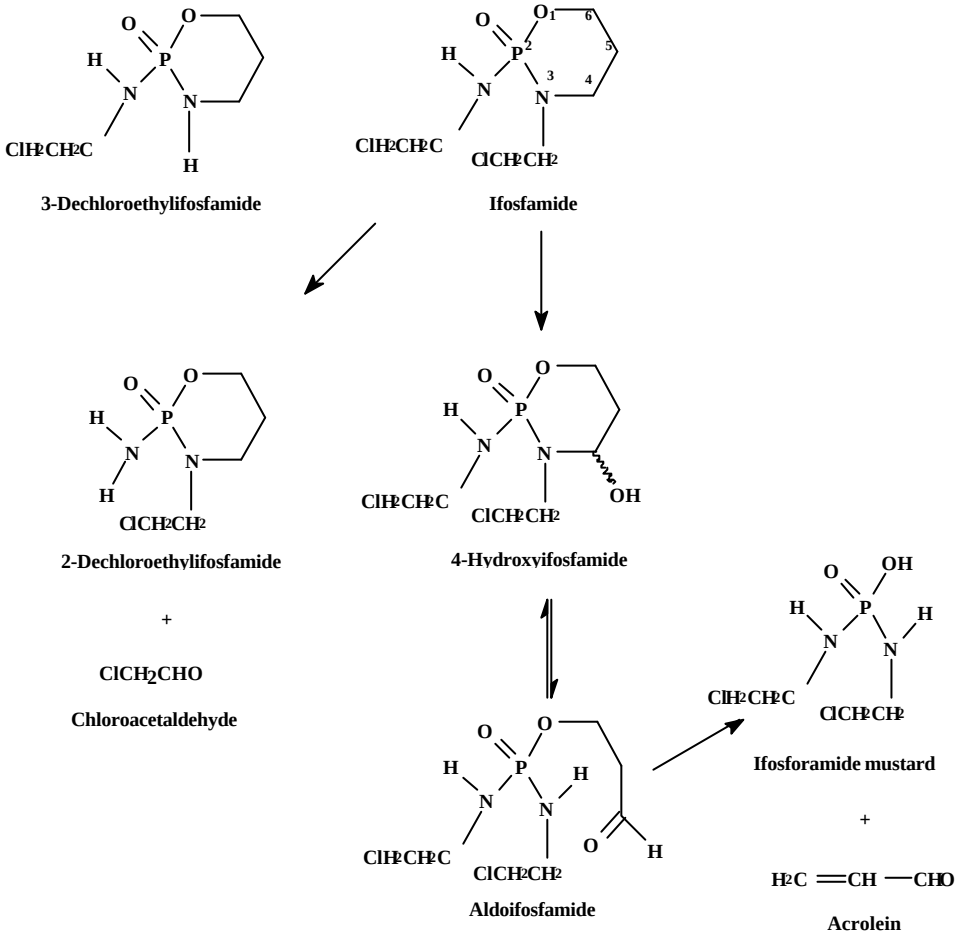

Figure 1. *Metabolism of prodrug ifosfamide with activation through 4-hydroxyifosfamide to the alkylating ifosforamide mustard and deactivation to 2- and 3-dechloroethylifosfamide.*

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide (Holoxan®, Ifex®), is commonly used for the treatment of various solid tumours, soft tissue sarcomas and haematological malignancies in adults and children.[1] Ifosfamide is a prodrug which needs activation via the cytochrome P450 enzymatic system in the liver to 4-hydroxyifosfamide. 4-Hydroxyifosfamide exists in equilibrium with its tautomeric form aldoifosfamide, which converts into the final alkylating agent ifosforamide mustard with concomitant acrolein release. Ifosfamide is also deactivated to the non-cytotoxic compounds 2-dechloroethylifosfamide and 3-dechloroethylifosfamide with simultaneous release of the potentially neurotoxic chloracetaldehyde.[1] The metabolism of ifosfamide is depicted in figure

1. The measurement of ifosfamide and metabolites has mostly been limited to plasma.[1] Recently, Highley et al. reported a strong preference of ifosforamide mustard, the active anti-tumour metabolite for the erythrocytes.[2] They observed that erythrocytes from five patients treated with ifosfamide contained as much as 77% of the total whole blood concentration of ifosforamide mustard, resulting in a ratio of the areas under the concentration-time curve of erythrocytes over plasma ($AUC_{e/p}$) of 2.7. This indicated that the relationship between concentrations in the plasma compartment and associated with erythrocytes may not be fixed and the two compartments could behave rather differently. Consequently, drug concentration-time profiles in whole blood and plasma could differ, thereby yielding different values for the pharmacokinetic parameters. To support ongoing pharmacokinetic and pharmaco-dynamic studies of ifosfamide, the possibility of any preferential distribution of ifosfamide and its metabolites to erythrocytes must be assessed.[3] The present work deals with the *in vitro* and *in vivo* distribution of ifosfamide, 2- and 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard in erythrocytes.

Materials and methods

Chemicals

Ifosfamide, 2- and 3-dechloroethylifosfamide, ifosforamide mustard and 4-hydroperoxy-ifosfamide were a kind gift of Dr J. Pohl, ASTA Medica AG (Frankfurt, Germany). Acetonitrile and methanol (HPLC supra-gradient) were purchased from Biosolve (Val-kenswaard, The Netherlands). 0.9 % Sodium chloride solution (NaCl) was obtained from the Slotervaart Hospital Pharmacy (Amsterdam, The Netherlands). Ethyl acetate (p.a. grade), anhydrous potassium dihydrogen phosphate (suprapure grade) were obtained from Merck (Darmstadt, Germany). Semicarbazide hydrochloride (p.a. grade) was purchased from Acros (Geel, Belgium). A 2 M semicarbazide solution (SCZ) for stabilising 4-hydroxyifosfamide was made and adjusted to pH 7.40 with 1 M HCl and 1 M NaOH prior to use. Semicarbazide is a derivatising agent for 4-hydroxyifosfamide yielding an ultra-violet detectable chromophore. Moreover, 4-hydroxyifosfamide is trapped by this derivatization and can no longer spontaneously decompose to ifosforamide mustard.[4] A 2 M semicarbazide with 1 M NaCl solution (SCZ/NaCl) for stabilising ifosforamide mustard was made and adjusted to pH 7.40 with 1 M HCl and 1 M NaOH, prior to use.[5] Distilled water was used throughout. Blank plasma, erythrocytes and heparinized whole blood were collected from healthy volunteers in the Slotervaart Hospital (Amsterdam, The Netherlands).

Preparation of standards and solutions

4-Hydroperoxyifosfamide was used as a substitute for 4-hydroxyifosfamide. In aqueous solution, 4-hydroperoxyifosfamide rapidly liberates 4-hydroxyifosfamide/aldoifosfamide and hydrogen peroxide. Fresh solutions of 4-hydroxyifosfamide were prepared immediately prior to use by dissolving an equimolar amount of 4-hydroperoxyifosfamide in distilled water. Dissolution was accelerated by ultra-sonification. Ifosforamide mustard was also prepared freshly for each experiment. Four separate *in vitro* experiments were performed. Experiment I

investigated the distribution of ifosfamide, 2- and 3-dechloroethylifosfamide between plasma and erythrocytes. 3 ml Venous blood at 37°C was added to 30 µl of a 50, 500 and 5000 µg/ml solution of ifosfamide, 2- and 3-dechloroethylifosfamide in a 0.9% NaCl solution. By using isotonic NaCl solutions undesired haemolysis of the erythrocytes by the stock solutions could be prevented. Next, 500 µl samples were aliquoted in six Eppendorf cups. The final drug concentrations in whole blood were 0.5, 5.0 and 50 µg/ml ifosfamide, 2- and 3-dechloroethylifosfamide. Experiment II investigated the distribution of 4-hydroxyifosfamide between plasma and erythrocytes. 12 ml Venous blood at 37°C was added to 120 µl of a 25 and 250 µg/ml solution of 4-hydroxyifosfamide in a 0.9 % NaCl solution. Next, 2000 µl samples were aliquoted in six Eppendorf cups. The final drug concentrations in whole blood were 0.25 and 2.5 µg/ml 4-hydroxyifosfamide. Experiment III investigated the distribution of ifosforamide mustard between plasma and erythrocytes. 6 ml Venous blood at 37°C was added to 60 µl of a 100 and 500 µg/ml solution of ifosforamide mustard in a 0.9 % NaCl solution. Next, 1000 µl samples were aliquoted in six Eppendorf cups. The final drug concentrations in whole blood were 1.00 and 5.00 µg/ml ifosforamide mustard. Experiment IV investigated the spontaneous conversion of 4-hydroxyifosfamide to ifosforamide mustard and subsequent distribution of ifosforamide mustard between plasma and erythrocytes. 6 ml Venous blood at 37°C was added to 60 µl of a 25 and 250 µg/ml solution of 4-hydroxyifosfamide in a 0.9 % NaCl. Next, 2000 µl samples were aliquoted in six Eppendorf cups. The final drug concentrations in whole blood were 0.25 and 2.5 µg/ml 4-hydroxyifosfamide. All venous blood samples were carefully mixed by gently tilting the tubes several times before they were aliquoted. All experiments were executed in triplicate. Since three bioanalytical methods with different stabilization and/or derivatization steps were applied, the *in vitro* experiments were not conducted simultaneously.

Incubation and sample pre-treatment

The samples were placed in a thermostatically controlled waterbath at 37°C. At various time-points (0, 5, 10, 20, 30 and 60 min) samples were taken and placed on ice water followed by immediate centrifugation for 2 min at 3,000 g at 4°C. After centrifugation the plasma layer was separated from the erythrocyte layer. The buffy coat with the leukocytes was removed carefully with sufficient margins and discarded, followed by transferring the erythrocytes. In order to exclude interference of haemolysis, a 50 µl plasma sample was used to determine the percentage haemolysis. In experiment I the ifosfamide, 2- and 3-dechloroethylifosfamide spiked samples were aliquoted in three 50-µl volumes of plasma and 50-µl volumes of the erythrocytes. In experiment II the 4-hydroxyifosfamide samples were aliquoted in two 250-µl volumes of plasma and two 250-µl volumes of the erythrocytes. In experiment III the ifosforamide mustard spiked samples were aliquoted in a 200-µl volume of plasma and a 200-µl volume of erythrocytes. In experiment IV the 4-hydroxyifosfamide spiked samples were aliquoted in a two 200-µl volume of plasma and a two 200-µl volume of erythrocytes for ifosforamide mustard determination. Immediately after incubation and matrix separation, samples containing 4-hydroxyifosfamide and ifosforamide mustard were stabilized by addition of 25 µl SCZ or 20 µl SCZ/NaCl, respectively, followed by vortex mixing for 15 sec. All samples were stored at -70°C pending analysis.

Determination of haemolysis

Interferences due to possible haemolysis have been investigated as follows: a volume of 2.5 ml distilled water was added to 25 µl venous blood yielding full haemolysis (100% standard sample) and 2.5 ml 0.9% NaCl was added to 25 µl venous blood with no haemolysis (0% standard sample). To check for haemolysis during the distribution experiments, a volume of 2.5 ml 0.9% NaCl was added to 25 µl plasma samples. If haemolysis of the erythrocytes would have occurred, erythrocyte cell-fragments would cause increased absorbance of the plasma matrix. The resulting mixture was mixed by gently tilting the tube several times and subsequent centrifugation for 10 min at 3,000 g. The absorption of all supernatants was measured at 540 nm. The 0 and 100% standard samples were measured in triplicate and the plasma samples were measured in duplicate. The percentage haemolysis was calculated by the ratio of the absorbance of the plasma sample (A_{plasma}) corrected for the background absorbance of 0.9% NaCl (A_{NaCl}) divided by the absorbance of 100% haemolysed venous blood (A_{water}) again corrected for the background absorbance of 0.9% NaCl, as described by equation 1.

$$\frac{A_{plasma} - A_{NaCl}}{A_{water} - A_{NaCl}} \times 100\% \qquad \text{(eq. 1)}$$

Patients

Venous whole blood samples of seven patients receiving 9 g/m²/72 hr intravenous ifosfamide treatment for soft tissue sarcoma were taken at 0, 3, 10, 20, 24, 34, 48, 58, 68, 72, 73, 76, 80 and 92 hours after start of the infusion. The samples were collected in 10-ml heparinized tubes and immediately cooled by placing them on ice water. The samples were then centrifuged without delay for 5 min with 1,000 g at 4°C. Plasma was transferred and the buffy coat was discarded, followed by transferring the erythrocytes. Both plasma and erythrocytes were aliquoted out for ifosfamide and metabolite analysis. Stabilization followed if required. Samples were stored at -70°C pending analysis of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide.

Analytical methods

Ifosfamide, 2- and 3-dechloroethylifosfamide were measured using gas chromatography with flame ionization nitrogen/phosphorous detection.[6] The samples were prepared by alkalinized liquid-liquid extraction (LLE) with ethyl acetate. Lower limit of quantification (LLQ) was 0.050 µg/ml for ifosfamide, 2- and 3-dechloroethylifosfamide. 4-hydroxyifosfamide was quantified using reversed phase high-performance liquid chromatography (RP-HPLC) with ultra-violet (UV) detection at 230 nm after LLE with ethyl acetate as reported previously.[4] LLQ was 0.100 µg/ml 4-hydroxyifosfamide. Ifosforamide mustard was determined by using RP-HPLC with UV detection at 280 nm.[5] This method allowed accurate determination of ifosforamide mustard in plasma, but not in erythrocytes due to interfering endogenous compounds. Samples were pre-treated by derivatization with diethyldithiocarbamate in phosphate buffer

after stabilising with SCZ/NaCl, followed by LLE with acetonitrile. LLQ was 0.100 µg/ml ifosforamide mustard. Since separate analytical methods were used the distribution experiments were also performed in separate series. Furthermore, by combining ifosfamide, 2- and 3-dechloroethylifosfamide in one experiment and 4-hydroxyifosfamide and ifosforamide mustard in separate experiments, specific conditions could be included for optimal stabilization of the latter two compounds. Concentrations of the analytes were measured in duplicate using standard calibration curves in the same matrix. Recovery of ifosfamide and its metabolites in the *in vitro* experiments was calculated according to equation 2,

$$\text{Recovery} = \frac{C_p \times (1-H) + C_e \times (H)}{C_b \times DF} \times 100\% \qquad \text{(eq. 2)}$$

in which C_p, C_e and C_b are the plasma, erythrocyte (blood cells) and nominal blood concentration, respectively. H is the haematocrit (volume of blood cells) and DF is the dilution factor (1-[volume of stock solution/volume of blood]).

Pharmacokinetic analysis

The mean partition coefficient value ($P_{e/p}$) of erythrocytes over plasma was used as an indicator for any preferential distribution to erythrocytes. $P_{e/p}$ was calculated by the ratio of the erythrocyte and plasma concentration. Ratios of the areas under the erythrocytes and plasma concentration-time curves ($AUC_{e/p}$) of the patients were calculated using a validated population approach.[3]

Results

Analytical methods

The *in vitro* experiments used a clinically relevant concentration range for each compound. The haemolysis during the distribution experiments over plasma and erythrocytes was always less than 3%. The haematocrit was determined at 44%. Mean (±sem) recoveries of ifosfamide, 2- and 3-dechloroethylifosfamide were 85.5±0.7%, 93.5±0.8% and 97.0±0.8%, respectively. 4-Hydroxyifosfamide was subject to autocatalytic degradation. At the beginning of the experiment recovery was 97.9±6.4%, but after 60 minutes recovery was only 0.7±0.2%.

In vitro distribution

In vitro equilibrium was obtained within 1 minute after drug addition (approximate time between spiking of the blood and sample collection at t=0). The mean (±sem) *in vitro* $P_{e/p}$s of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were 0.75±0.01, 0.62±0.09, 0.76±0.10 and 1.38±0.04, respectively (table 1).

Table 1. *Partition coefficients ($P_{e/p}$) and ratios of the area under the erythrocyte and plasma concentration-time curves ($AUC_{e/p}$) of ifosfamide (IF), 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF).*

	in vitro $P_{e/p}$ Mean (±sem)	*in vivo* $P_{e/p}$ Mean (±sem)	*in vivo* $AUC_{e/p}$ Mean (±sem)
IFO	0.75±0.01	0.81±0.03	0.96±0.03
2DCE	0.62±0.09	0.73±0.03	0.87±0.07
3DCE	0.76±0.10	0.93±0.05	0.98±0.06
4OHIF	1.38±0.04	0.98±0.09	1.34±0.39

No trends with time or in concentration were observed, as presented in figure 2. $P_{e/p}$s of ifosfamide, 2- and 3-dechloroethylifosfamide were always less than one. In contrast, $P_{e/p}$ of 4-hydroxyifosfamide was always higher than one. Not all 4-hydroxyifosfamide concentrations could be determined, because they were below the LLQ. In figure 3 the autocatalytic degradation of 4-hydroxyifosfamide in plasma and erythrocytes is depicted. No difference in degradation rate was observed between plasma or erythrocytes as the profiles decayed in parallel. Mean (±sem) elimination half-lives in plasma and erythrocytes were 9.0±0.3 and 9.1±0.4 min, respectively. The plasma concentrations of ifosforamide mustard are depicted in figure 4. During incubation with 1.0 µg/ml ifosforamide mustard, plasma concentrations did not exceed 1.0 µg/ml. During incubation with 5.0 µg/ml ifosforamide mustard, plasma concentrations were higher than 5.0 µg/ml but declined towards 5.0 µg/ml after 20 minutes. No degradation of ifosforamide mustard was observed during the incubation. The formation of ifosforamide mustard from 4-hydroxyifosfamide is also depicted in figure 4.

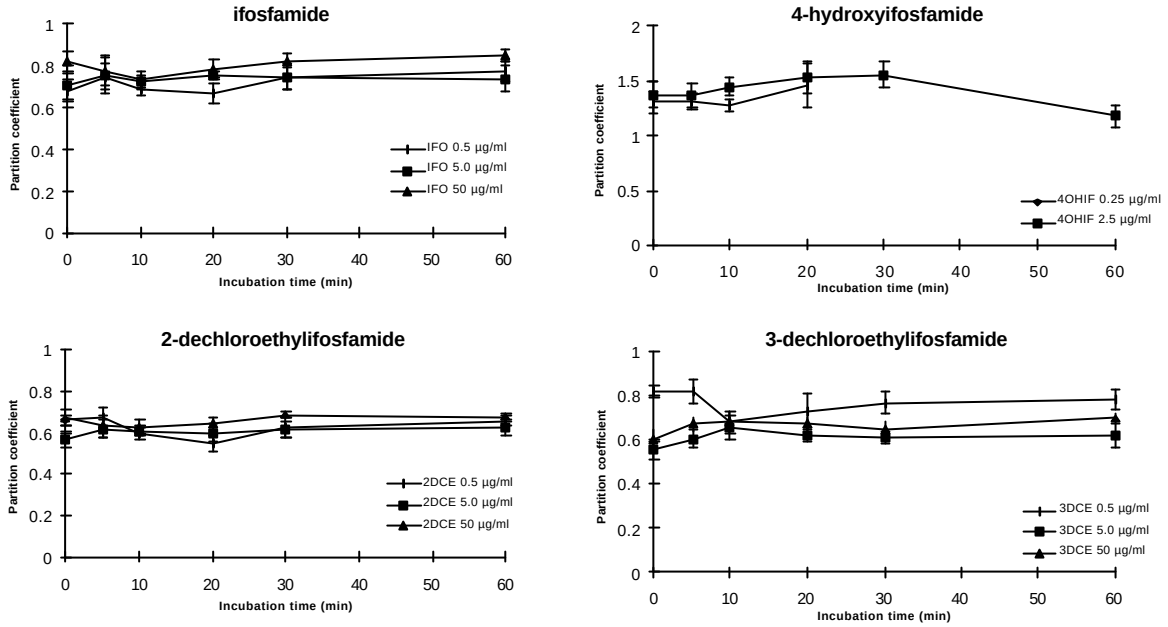

Figure 2. *Mean (±sem) in vitro partition coefficients of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide during incubation at 37°C.*

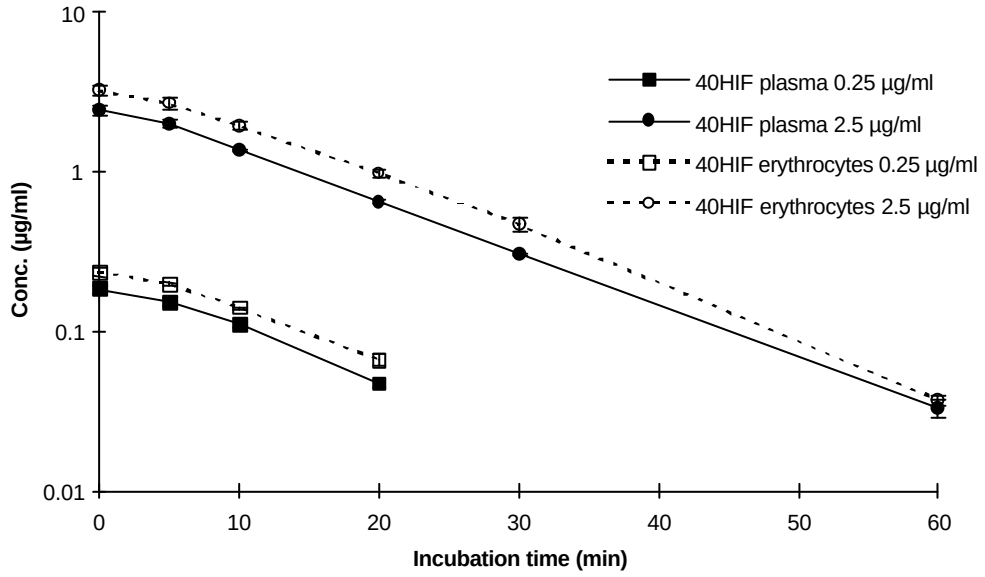

Figure 3. *Plasma and erythrocyte concentrations of 4-hydroxyifosfamide (4OHIF) during incubation at 37°C.*

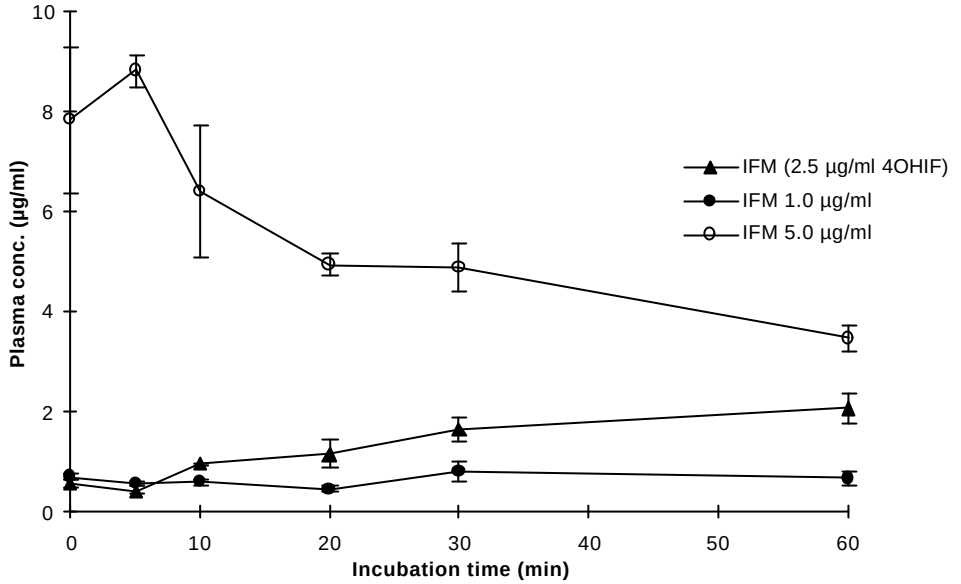

Figure 4. *Plasma concentrations of ifosforamide mustard (IFM) during incubation of ifosforamide and 4-hydroxyifosfamide (4OHIF) at 37°C.*

Incubation with 0.25 µg/ml 4-hydroxyifosfamide did not yield ifosforamide mustard plasma concentrations above the LLQ. Incubation with 2.5 µg/ml 4-hydroxyifosfamide yielded an equimolar ifosforamide mustard plasma concentration of 2.0 µg/ml. Thus, complete conversion of 4-hydroxyifosfamide to ifosforamide mustard without further degradation of ifosforamide mustard was observed after incubating for 60 minutes at 37°C. The formation half-life of ifosforamide mustard was 28.1 min^{-1}.

Clinical pharmacokinetics

Typical plasma and erythrocyte concentration-time profiles of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide after a 72-hour continuous infusion of 9 g/m^2 ifosfamide are depicted in figure 5. The pharmacokinetics assessment in seven patients receiving this infusion resulted in 85, 86, 83 and 70 samples for ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide. Their mean (±sem) *in vivo* $P_{e/p}$s of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were 0.81±0.03, 0.73±0.03, 0.93±0.05 and 0.98±0.09, respectively (table 1). The plots of $P_{e/p}$s with time are depicted in figure 6. $P_{e/p}$s did not vary during the 72-hour ifosfamide infusion. However, during a brief period after cessation of the infusion a preference of ifosfamide and metabolites for the erythrocytes was observed. $P_{e/p}$s of ifosfamide, 2- and 3-dechloroethylifosfamide were always less than one, but the coefficient for 4-hydroxyifosfamide was always higher than one during the infusion. The mean (±sem) $AUC_{e/p}$s of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were 0.96±0.03, 0.87±0.07, 0.98±0.06, 1.34±0.39, respectively (table 1).

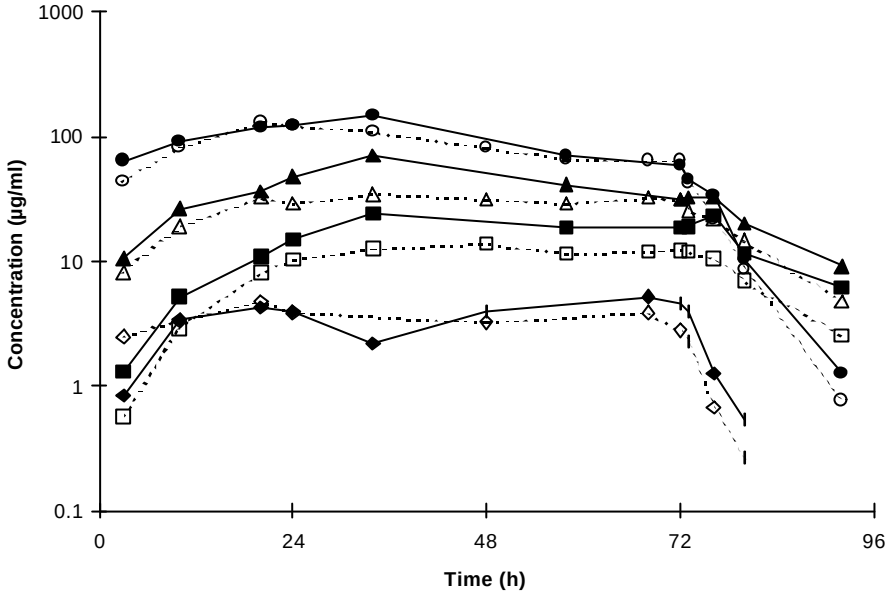

Figure 5. *Plasma (solid lines) and erythrocyte (dashed lines) concentrations of ifosfamide (circles), 2-dechloroethylifosfamide (squares), 3-dechloroethylifosfamide (triangles) and 4-hydroxyifosfamide (diamonds) in a typical patient receiving 9 g/m² ifosfamide in a 72-hour continuous i.v. infusion.*

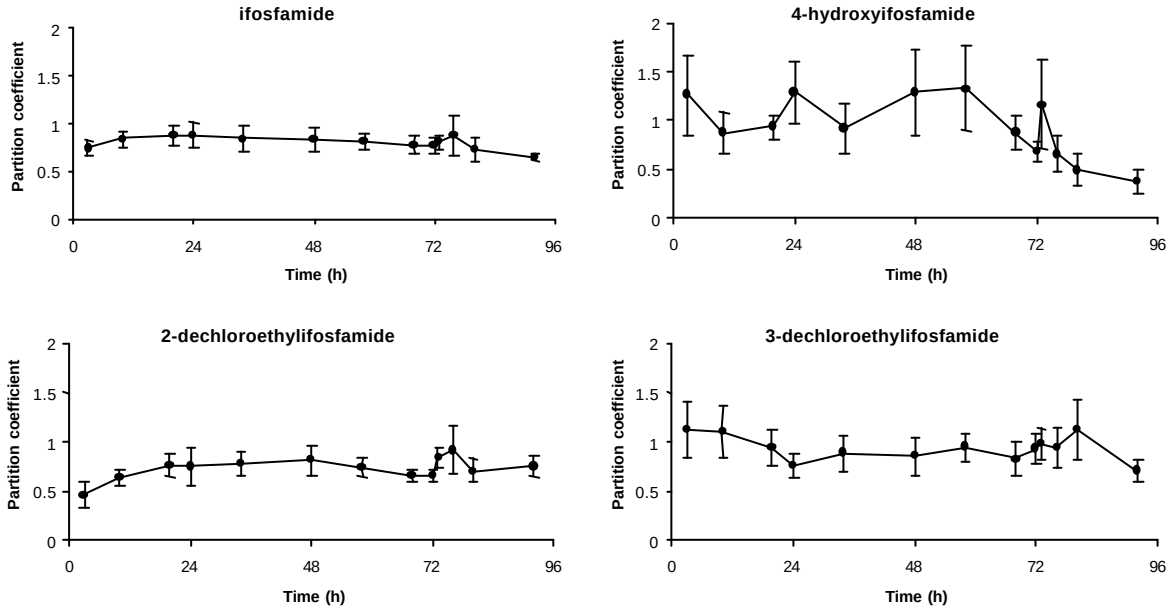

Figure 6. *Mean (±sem) in vivo partition coefficients of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide in seven patients receiving 9 g/m² ifosfamide in a 72-hour continuous i.v. infusion.*

Discussion

So far, the pharmacokinetics of ifosfamide and metabolites have mostly been defined with reference to drug in plasma. Considering that erythrocytes comprise about 40-50% of the whole blood volume, it is necessary to investigate possible differences in kinetics in plasma and erythrocytes. We performed *in vitro* and *in vivo* experiments to investigate possible kinetic differences of ifosfamide, 2- and 3-dechloroethylifosfamide, 4-hydroxy-ifosfamide and ifosforamide mustard in plasma and erythrocytes.

Drug measurement studies in erythrocytes must safeguard against possible lower reproducibility and accuracy in handling this matrix. The method used in the current study involved separation of erythrocyte sediments by centrifugation at 3,000 g. Recently, a novel device (MESED) was introduced for erythrocyte separation.[7] The investigators pointed out that with regular centrifugation plasma is possibly still trapped between erythrocytes which could interfere with the determination of possible accumulation of a compound in erythrocytes. However, the reduction in trapped plasma in the erythrocyte layer using MESED was only 1.9% with 10,000 g (from 3.9 to 2.0%).[7] Furthermore, this labour-intensive and costly method required three centrifugation steps, impeding routine use in clinical studies and possibly allowing degradation of unstable metabolites. Since the gain in erythrocyte purity was not considered significant, normal centrifugation was assumed to be sufficient in matrix separation.

The haemolysis during the *in vitro* experiments was always less than 3%. Thus, the erythrocytes remained intact during the experiment. This implies that $P_{e/p} < 1$ of a compound could not be caused by a transfer from the erythrocyte to the plasma compartment as a consequence of haemolysis. It was *in vitro* confirmed that total drug recovery of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide (t=0) from erythrocyte and plasma fraction were within the accuracy of the analytical methods.[4-6] Lack of degradation of ifosforamide mustard could be assumed because equimolar conversion of 4-hydroxyifosfamide to ifosforamide mustard was observed. The nominal blood concentration of 2.5 µg/ml 4-hydroxyifosfamide could maximally be converted to 2.0 µg/ml ifosforamide mustard. This concentration was reached after 60 minutes, thus complete conversion without degradation was observed.

Highley and co-workers reported differences in ifosforamide mustard distribution between plasma and erythrocytes in patients receiving ifosfamide.[2,8] An integrated bioanalytical method using gas chromatography with mass detection was applied in their studies.[9] They determined ifosfamide, 2- and 3-dechloroethylifosfamide, ifosforamide mustard, carboxyifosfamide, ketoifosfamide and two metabolites of ifosforamide mustard but not 4-hydroxyifosfamide. The ifosforamide mustard as determined by their assay represented the sum of free ifosforamide mustard and 4-hydroxyifosfamide because no stabilization of 4-hydroxyifosfamide was performed. They observed higher concentrations in the erythrocytes of ifosforamide mustard and carboxyifosfamide (a metabolite of 4-hydroxyifosfamide), but not with ifosfamide, 2- and 3-dechloroethyl-ifosfamide.

In accordance with the findings of Highley and co-workers the present study shows that to a large extent ifosfamide and metabolites are located in plasma: drug distribution to the erythrocyte compartment ranged from about 38% for 2-dechloroethylifosfamide to 43% for

ifosfamide and was stable over a wide range of clinically relevant concentrations. 4-Hydroxyifosfamide showed affinity for the erythrocytes with 58% of the total drug concentration associated with this fraction. Distribution to erythrocytes was very rapid because no change in $P_{e/p}$s was observed between the start and the end of the incubation, indicating that the compounds can enter the erythrocyte compartment freely. This was confirmed by the parallelism in *in vitro* erythrocyte and plasma concentration profiles. The pharmacokinetics of 4-hydroxyifosfamide are expected to be related with efficacy of ifosfamide therapy.[1] The autocatalytic degradation of 4hydroxyifosfamide was equal in plasma and erythrocytes, indicating that no differences between pharmacokinetic parameters of 4-hydroxyifosfamide are expected when using plasma or erythrocytes.

A concentration dependent difference in distribution of ifosforamide mustard was observed. Incubation with 1.0 µg/ml ifosforamide mustard did not demonstrate a particular affinity for the erythrocytes. However, incubation with 5.0 µg/ml ifosforamide mustard demonstrated increased plasma concentrations of ifosforamide mustard during the first 20 minutes. It can be hypothesized that the more hydrophilic ifosforamide mustard in comparison to the other analytes is less capable of penetrating the erythrocyte. Furthermore, ifosforamide mustard is highly ionized and hence does not readily cross membranes.[10] To reach a distribution-equilibrium of ifosforamide mustard will require time due to this concentration dependent phenomenon. This phenomenon was not observed during the conversion of 4-hydroxyifosfamide to ifosforamide mustard, possibly due to the lower incubation concentration. Furthermore, a relatively lower amount of ifosforamide mustard will be exposed to the erythrocytes during conversion. The *in vivo* experiment was evidently done with all compounds simultaneously, thus incorporating possible interactions between the metabolites that could influence their distribution between plasma and erythrocytes. The *in vivo* $P_{e/p}$s and $AUC_{e/p}$ were in accordance with the *in vitro* findings (table 1). The apparent shift in affinity towards the erythrocytes of ifosfamide and its metabolites during the elimination phase could be explained by a lack of equilibrium between plasma and erythrocytes. However, this transient shift was only minor and therefore probably not clinically relevant.

The lack of *in vitro* affinity for the erythrocyte of ifosforamide mustard is apparently in disagreement with the findings in patients by Highley et al.[2] However, 4-hydroxyifosfamide exhibited *in vitro* and *in vivo* affinity for erythrocytes. Moreover, the pharmacokinetics of 4-hydroxyifosfamide in the two compartments did not differ. It can be hypothesized that relatively more 4-hydroxyifosfamide will reside in the erythrocyte compartment. Consequently, relatively more ifosforamide mustard will be formed in this compartment, thus corroborating the findings of Highley and co-workers.[2]

It is concluded that ifosfamide and metabolites rapidly reach distribution equilibrium between erythrocytes and plasma, with ifosforamide mustard being the slowest. Drug distribution to the erythrocyte fraction ranged from about 38% for 2-dechloroethyl-ifosfamide to 58% for 4-hydroxyifosfamide and was stable over a wide range of clinically relevant concentrations. A strong parallelism in the erythrocyte and plasma concentration profiles was observed for all compounds, indicating that no differences will arise in the assessment of pharmacokinetic parameters using either matrix. Thus, pharmaco-kinetic assessment using only plasma sampling can yield direct, accurate and relevant relationships with efficacy and toxicity in patients treated with ifosfamide.

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Chapter 3.1

Evaluation of the autoinduction of ifosfamide metabolism by a population pharmacokinetic approach using NONMEM

Summary

This study investigated the population pharmacokinetics of ifosfamide in 15 patients treated for soft tissue sarcoma with 9 or 12 g/m² ifosfamide by means of a 72-hour continuous intravenous infusion. A model was developed using non-linear mixed effect modelling (NONMEM) to describe the non-linear pharmacokinetics of ifosfamide by linking the ifosfamide plasma concentrations to the extent of the autoinduction. The proposed model revealed the effect of autoinduction on the disposition of ifosfamide. The initial clearance, volume of distribution, rate constant for enzyme degradation, induction half-life of the enzyme and the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation were estimated at 2.94±0.27 L· h^{-1}, 43.5±2.9 L, 0.0546±0.0078 h^{-1}, 12.7 h and 30.7±4.8 µM, respectively. Interindividual variabilities of initial clearance, volume of distribution, rate constant for enzyme degradation were 24.5, 23.4 and 22.7%, respectively. Proportional and additive variability not explained by the model were 13.6% and 0.0763 µM, respectively. The absence of a lag time for the autoinduction of ifosfamide metabolism could be the result of an immediate inhibition of the enzymatic degradation of CYP3A4 by ifosfamide. By application of the autoinduction model individual pharmacokinetic profiles of patients were described with adequate precision. This model may therefore be used in the future development of a model to individualize dose selection in patients.

Introduction

Ifosfamide (N,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide, Holoxan®) is an alkylating oxazaphosphorine widely applied as anti-cancer agent in the treatment of various solid and haematological malignancies in adults and children.[1,2] Ifosfamide is a prodrug requiring activation to 4-hydroxyifosfamide as shown in figure 1.

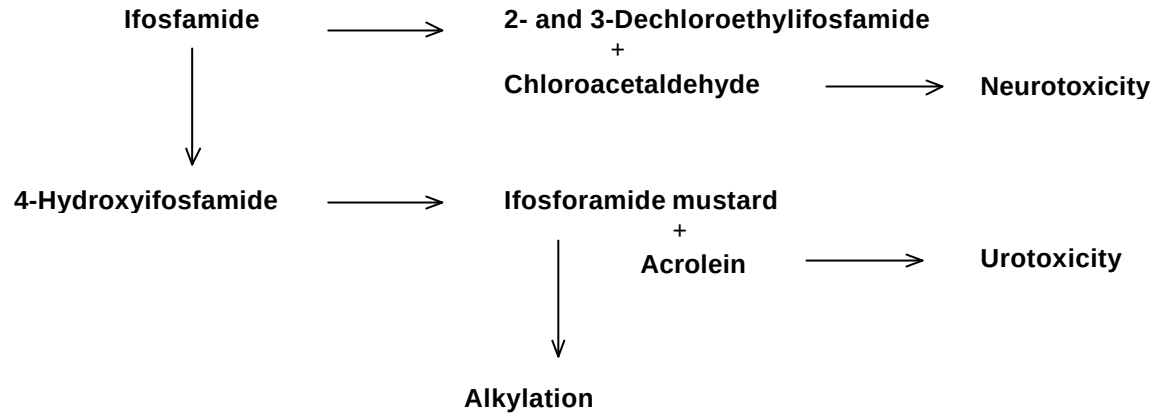

Figure 1. *Metabolism of the prodrug ifosfamide. Ifosfamide is activated through 4-hydroxyifosfamide to the ultimate alkylating agent ifosforamide mustard by ß-elimination of urotoxic acrolein. Ifosfamide is deactivated to 2- and 3-dechloroethylifosfamide yielding neurotoxic chloroacetaldehyde.*

After ß-elimination of the urotoxic acrolein from 4-hydroxyifosfamide, the alkylating ifosforamide mustard is formed.[3] Deactivation of ifosfamide leads to 2- and 3-dechloroethylifosfamide and the release of the potentially neurotoxic chloroacetaldehyde. Both activation (hydroxylation) and deactivation (dechloroethylation) are mediated by the cytochrome P450 3A4 (CYP3A4) iso-enzyme.[4,5] Metabolism of ifosfamide is an autoinducible enzymatic process, resulting in increased clearance over time.[6]

During the last decades several methods have been proposed for the description of the pharmacokinetics of ifosfamide and its metabolites.[7] Allen & Creaven and Lind et al. used a two-compartment pharmacokinetic model for describing the pharmacokinetics of ifosfamide.[8,6] Nelson et al. proved a one-compartment pharmacokinetic model to be superior for 1.5 to 2.4 g/m^2 in 30 min. intravenous (i.v.) infusion but not when dosed 3.8 to 5 g/m^2.[9] Boddy et al. used a model independent approach for describing ifosfamide pharmacokinetics.[1] All these methods were developed to describe concentration-time profiles of infusions of short duration (1-3 hours) and did not take into account the effect of the autoinduction on the pharmacokinetics of ifosfamide. Prasad et al. and Boddy et al. were the first to report models, enabling an adequate description of the concentration-time data for ifosfamide infusions of long duration of up to 72 hours.[10,11] Their models included a lag time before the development of autoinduction started and described the increase of clearance of ifosfamide over time.

Large interpatient variability in clinical toxicity and response rates is observed during ifosfamide treatment.[12] This could possibly be explained by interpatient differences in ifosfamide pharmacokinetics e.g. autoinduction. In this study we aimed to develop a non-linear pharmacokinetic model for ifosfamide, by which all relevant pharmacokinetic parameters including the rate and extent of autoinduction over time could be estimated. Our future aim is to investigate the possibility of therapeutic drug monitoring (TDM) of ifosfamide.

Therefore, a population pharmacokinetic approach was selected because this allowed assessment of interindividual and residual variability necessary for TDM. Furthermore, a simulation was performed to assess the influence of autoinduction on the pharmacokinetics of ifosfamide.

Methods

Patient population and treatment

Patients treated for various types of soft tissue sarcoma with single agent high-dose ifosfamide in an open non-randomized phase II trial, were subjected to pharmacokinetic sampling after written informed consent was obtained. Six patients received 12 g/m² ifosfamide as a 72-hour continuous i.v. infusion once every 4 weeks. Due to unacceptable central neurotoxicity in this group the following nine patients received a decreased dose of 9 g/m²/72 h. This resulted in individual total doses ranging from 10.3 to 23.5 g ifosfamide. Supportive care consisted of mesna and extensive hydration to prevent haemorrhagic cystitis and bicarbonate to prevent acidosis. Anti-emetics and methylene blue (a neurotoxicity antidote) were given when indicated. Additional co-medication consisted of 21 different drugs with a mean of seven per patient, including anti-coagulants, H_2 receptor antagonists, glucocorticosteroids and tricyclic anti-depressants.

Pharmacokinetic sampling and analysis

Whole blood samples were drawn at 0, 3, 10, 20, 24, 34, 48, 58, 68, 72, 73, 76, 80 and 92 hours after start of the infusion. Following plasma separation the samples were stored at -70°C pending analysis. After alkalinized liquid-liquid extraction with ethyl acetate, ifosfamide was determined using gas-chromatography with nitrogen-phosphorous detection.[13] The method was validated resulting in an accuracy of 103.2, 99.9, 104.5, 102.0 and 93.3%, an intra-assay precision of 5.5, 0.8, 1.8, 2.0 and 2.9% and an inter-assay precision of 3.5, 2.9, 3.6, 2.6 and 5.1% at concentrations of 0.192, 0.383, 0.958, 38.3 and 383 µM ifosfamide, respectively.

Model development

Ifosfamide plasma concentration-time profiles were described by a one-compartment model as depicted in figure 2. The change of the amount of ifosfamide in the central compartment (A_1, µmol) over time was described by equation 1,

$$\frac{dA_1}{dt} = R - \left(CL_{app} \times C_p\right) \qquad \text{(eq. 1)}$$

in which R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL_{app} (L· h^{-1}) is the apparent ifosfamide clearance and C_p (µmol· L^{-1}) is the plasma concentration in compartment 1, which is equal to A_1/V with V (L) as the volume of distribution of ifosfamide. CL_{app} is defined by equation 2.

$$CL_{app} = CL \times A_2 \qquad \text{(eq. 2)}$$

In this equation CL ($L \cdot h^{-1}$) is the initial clearance of ifosfamide and A_2 is an amount of enzyme in the hypothetical compartment 2 as depicted in figure 2. The change in A_2 over time is described by equation 3,

$$\frac{dA_2}{dt} = K_{enz,in} - K_{enz,out} \times A_2 \times \left(1 - \frac{C_p}{C_p + IC_{50}}\right) \qquad \text{(eq. 3)}$$

in which $K_{enz,in}$ ($mol \cdot h^{-1}$) is the zero-order rate constant for enzyme formation/activation, $K_{enz,out}$ (h^{-1}) is the first-order rate constant for enzyme degradation/inactivation and IC_{50} (µM) is the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation. Before treatment with ifosfamide (t=0) steady-state levels of the enzyme were assumed. Hence, the change in enzyme amount was zero. $K_{enz,in}$ was equal to $K_{enz,out}$ times the amount of enzyme at t=0 ($A_{2\ t=0}$). By defining $A_{2,t=0}$ at a value of 1, $K_{enz,in}$ could be substituted for $K_{enz,out}$, yielding equation 4,

$$\frac{dA_2}{dt} = K_{enz,out} - K_{enz,out} \times A_2 \times \left(1 - \frac{C_p}{C_p + IC_{50}}\right) \qquad \text{(eq. 4)}$$

in which A_2 is the relative amount of enzyme. The induction half-life of the enzyme ($T_{½,enz}$, h) was calculated by dividing ln(2) by $K_{enz,out}$.

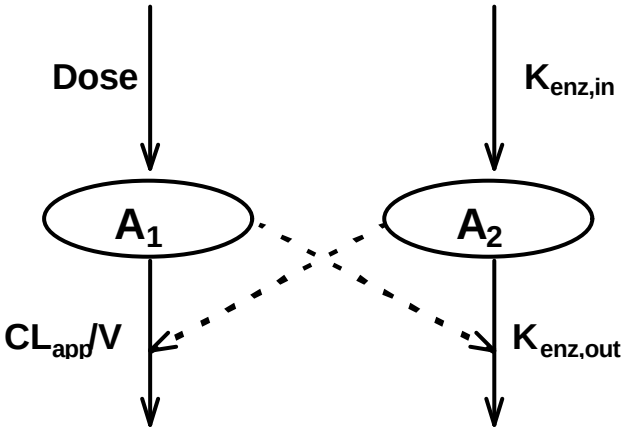

Figure 2. *Pharmacokinetic model of ifosfamide with autoinduction of its metabolism. The time profile of ifosfamide amount (A_1, µmol) in compartment 1 was described by an influx of dose and an efflux calculated by an apparent clearance (CL_{app}, $L \cdot h^{-1}$) divided by volume of distribution (V, L). The time profile of the relative amount of enzyme (A_2) in the hypothetical compartment 2 was determined by a rate constant for enzyme formation ($K_{enz,in}$) and a rate constant for enzyme degradation ($K_{enz,out}$). The dashed arrows represent the effect of the enzyme (A_2) on the elimination rate of ifosfamide (CL_{app}/V) and the effect of ifosfamide (A_1) on the degradation rate ($K_{enz,out}$) of the enzyme.*

Data analysis was performed using the non-linear mixed effect modelling programme (NONMEM) (double precision, version V, level 1.0) operated on an MS-DOS computer under FORTRAN90 Powerstation (version 4.0).[14] Population and individual estimates for CL, V, $K_{enz,out}$ and IC_{50} were obtained using a general non-linear population pharmacokinetic programme of NONMEM with first-order processes and non-stiff differential equations (ADVAN6 TOL=5).[15] The interindividual variability for all pharmacokinetic parameters was estimated with a proportional error model. The residual variability was modelled using a

combined additive plus proportional error model. The POSTHOC function of NONMEM enabled estimation of the individual pharmacokinetic parameters using a Bayesian approach taking both individual observations and population effects into account. The predictive performance of the model was assessed by plotting the population predicted (PRED) and individual Bayesian predicted estimates (IPRED) versus the observed concentrations (OBS). The effect of autoinduction on the dose-area under the curve (AUC) relationship was investigated by simulation. The profiles were simulated based upon the population parameters found.

Results

The patient population consisted of nine males and six females with a mean age of 49 years (ranging from 23 to 72) and a mean weight of 59 kg (ranging from 49 to 82). Tumour types were rhabdomyosarcoma (n=3), neurofibrosarcoma (n=1), osteosarcoma (n=3), synoviumsarcoma (n=2), leiomyosarcoma (n=4) and endometriumsarcoma (n=2).

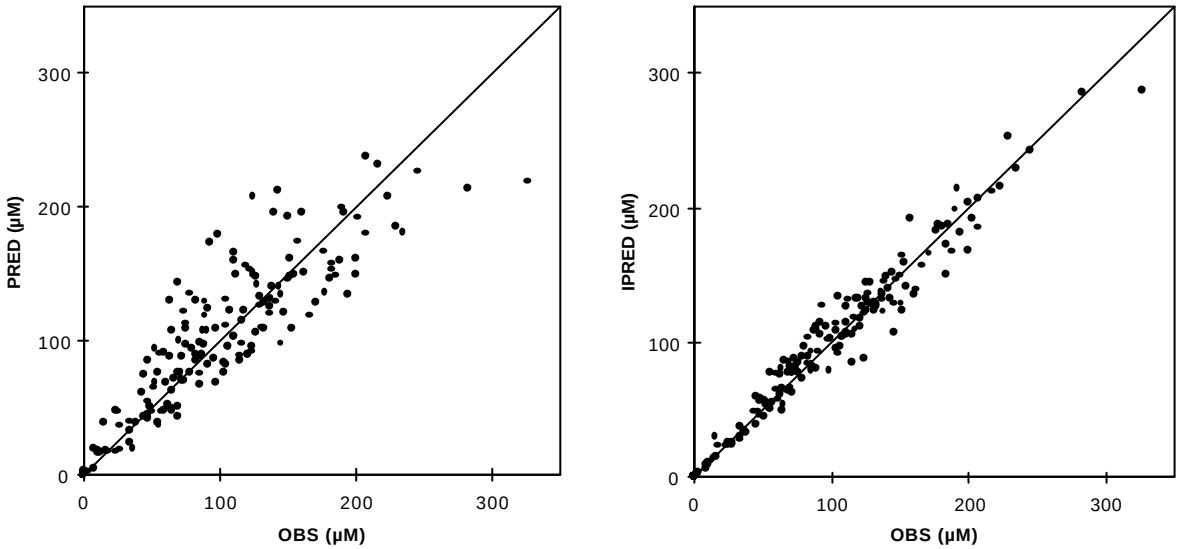

Figure 3. *The relationships between estimates (PRED) and individual Bayesian estimates (IPRED) versus observed (OBS) ifosfamide concentrations.*

Pharmacokinetic assessment of the 15 patients resulted in 176 concentration-time points. The predictive performance is represented in figure 3. By including individual estimates the predictive performance was increased. The individual predictions (IPRED) were symmetrically distributed around the line of unity, suggesting adequate model prediction. No outliers or trends could be observed in the concentration range or time range. The observed and Bayesian estimated pharmacokinetic profiles of all patients are represented in figure 4. Most patients received 72-hour infusions and showed initial build-up of ifosfamide plasma concentrations in the first 24 hours (reaching maximum concentrations), followed by a gradual decrease due to autoinduction. After 72 hours the infusion was stopped and elimination pharmacokinetics could be observed. The infusion in one patient was stopped after 48 hours, due to acute severe neurotoxicity. Therefore, the patient receiving only 66% of

the planned dose (open circles).

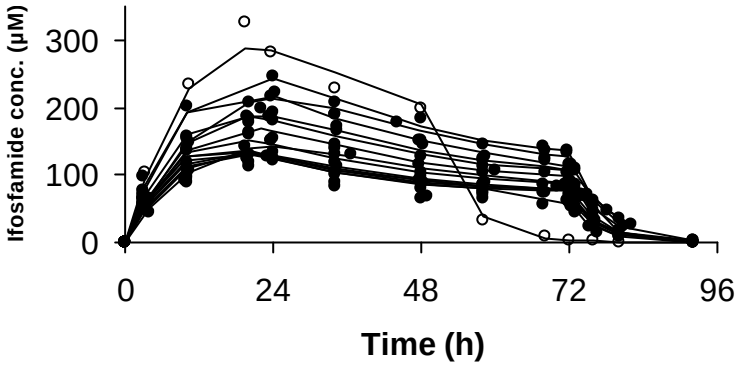

Figure 4. *The observed (dots) and Bayesian estimated (lines) ifosfamide concentrations vs. time for all patients (n=15). The open circles represent a patient who suffered from a neurotoxicity episode. The drug infusion for this patient was stopped after 48 hours.*

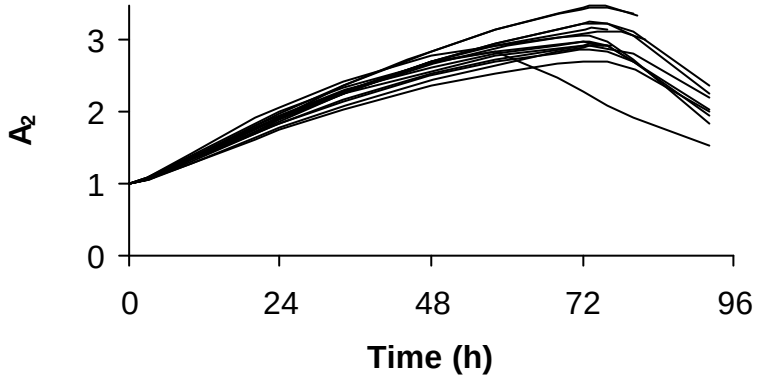

Figure 5. *Individual time profiles of the relative amount of enzyme (A_2) for all patients (n=15). The profiles were obtained on basis of the individual Bayesian parameter estimates provided by NONMEM. At the initiation of the therapy A_2 was 100%.*

The predicted relative amount of enzyme in the hypothetical compartment 2 is described in figure 5. The relative amount of enzyme increased over time during the infusion and decreased after cessation. Notably, during the first 24 hours for all patients the model predicted an average doubling of the relative enzyme amount. Estimates of pharmacokinetic population parameters, their standard error, their interindividual variability and the residual variability are presented in table 1.

Table 1. *Estimates of pharmacokinetic population parameters for ifosfamide with their standard error, interindividual variability and residual variability.*

Parameter	Mean	Standard Error	Interindividual Variability	Residual Variability
CL ($L \cdot h^{-1}$)	2.94	0.27	24.5%	
V (L)	43.5	2.9	23.4%	
$K_{enz,out}$ (h^{-1})	0.0546	0.0078	22.7%	
$T_{½,enz}$ (h)	12.7			
IC_{50} (µM)	30.7	4.8		

P.E.	13.6%
A.E. (µM)	0.0763

CL=initial ifosfamide clearance, V=volume of distribution of ifosfamide, $K_{enz,out}$=first-order rate constant for enzyme degradation, $T_{½,enz}$=induction half-life of the enzyme, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, P.E.=proportional residual error, A.E.=additive residual error.

The effect of autoinduction on the AUC after doubling of the dose is simulated in figure 6 and represented in table 2. This simulation shows that after increasing the dose from 10 to 20 g ifosfamide, the AUC was only 56% larger.

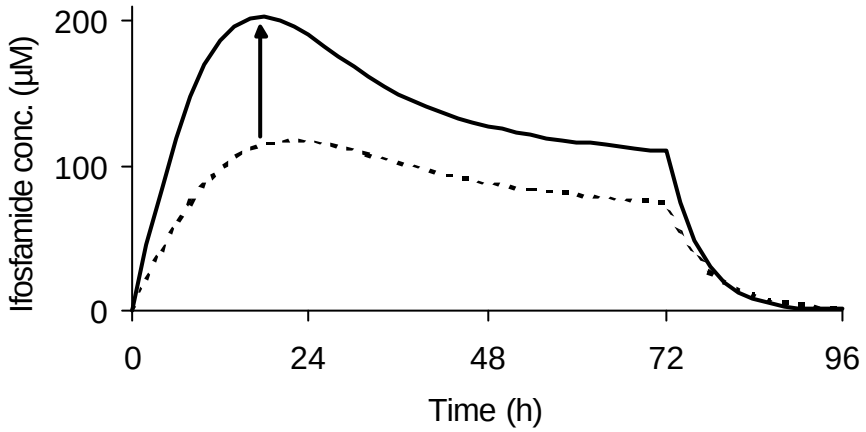

Figure 6. *The simulated pharmacokinetic profiles of ifosfamide after 10 g (dashed line) and 20 g (solid line) ifosfamide in a patient receiving a 72-hour continuous intravenous infusion, based upon population parameters found.*

Table 2. *The simulated effect of different doses of ifosfamide on the area under the plasma concentration-time curve (AUC).*

Ifosfamide dose (g/72 hr)	AUC (µM· h)
10	1.7
20	2.7
increase	56 %

Discussion

A population model of the pharmacokinetics of ifosfamide has been presented in which autoinduction was modelled by inhibition of the degradation of the enzyme responsible for ifosfamide metabolism. The exact physiological mechanism of the autoinduction of ifosfamide is unknown, but several mechanisms of drug induced metabolic changes have been reported for other compounds. For most of these drugs the amount of enzymes performing the metabolic breakdown is increased via *de novo* synthesis. For instance, the barbiturate pentobarbital induces nortriptyline metabolism by increasing translation of the DNA coding for

the enzyme and thereby increasing clearance of systemic nortriptyline. De novo synthesis of cytochrome P450 (CYP) by pentobarbital was observed after two days.[16] Rapid autoinduction of carbamazepine due to *de novo* synthesis was already demonstrated after 12-24 hours with a maximum after six weeks.[17] Autoinduction of ifosfamide has been observed within 24 hours after the start of the treatment.[18] However, it is unlikely that an alkylating agent like ifosfamide (which reduces DNA/RNA and protein synthesis) increases enzyme protein synthesis of CYP. Non-transcriptional induction mechanisms have been demonstrated to produce drug induced metabolic changes as well. Troleandomycin produced no increase in the rate of CYP3A4 protein synthesis, but it decreased the rate of the CYP3A4 protein degradation by interacting with specific catalytic enzymes.[19] This resulted in an increased pool of the CYP3A4 protein. An uncommon interaction of a drug with its metabolizing enzyme has been demonstrated for phenobarbital.[20] This interaction did not result in an increased synthesis of the enzyme, but was achieved by direct influence of the drug on the enzyme. By binding of the drug on a non-catalytic site of the enzyme, the catalytic ability increased due to an altered spin-state of the haemoprotein of the CYP.

Ifosfamide has been shown to reduce protein synthesis and could have a similar effect on the enzymes responsible for CYP3A4 degradation/inactivation, thus increasing ifosfamide clearance.[21] CYP3A4 performs both a desired hydroxylation and an undesired dechloroethylation of ifosfamide, making it a target for the development of a specific inhibition or heteroinduction regimens and thereby directing ifosfamide metabolism to a more favourable toxicity-efficacy profile. Although co-medication consisted of a total of 24 different drugs, none have been shown to be inhibitors or inducers of CYP3A4. Furthermore, no metabolic interactions between ifosfamide and any of these drugs have been reported.

Figures 3 and 4 indicate that the proposed population pharmacokinetic model was precise and without misspecifications and thus described the observed data well. Although population programmes such as NONMEM were designed to describe populations using sparse data, the development of population models requires fewer subjects if the pharmacokinetic data are rich. Figure 4 indicates that the patient suffering from severe neurotoxicity had the highest AUC between 0 to 48 hours. The CL and $K_{enz,out}$ of this patient were 2.25 L/h and 0.0471 h^{-1}, respectively. Population values ranged from 2.22 to 4.96 L/h and from 0.0439 to 0.0697 h^{-1}, respectively. Curiously, this patient had the second lowest $K_{enz,out}$ (a slow onset of autoinduction) and the second lowest CL of the subjects receiving a dose of 12 g/m². The relative low clearance of ifosfamide in this patient should theoretically result in a lower AUC of neurotoxic metabolites and less neurotoxicity. The latter is in contrast with our observations. It can therefore be concluded that the development of neurotoxicity cannot be predicted or explained on the basis of the pharmacokinetic parameters of ifosfamide alone.

Previously reported models of ifosfamide pharmacokinetics used a lag time before the start of autoinduction, because it was first noticed only after 24 hours.[10,11] Our model described the autoinduction as a gradually developing effect with a half-life of 12.7 hours. Figure 5 shows that our data could be modelled without a lag time. The absence of a lag time for the autoinduction of ifosfamide metabolism could be the result of direct inhibition of the enzymatic degradation of CYP3A4 by ifosfamide, rather than its *de novo* synthesis, which requires time. However, activation of CYP3A4 activity cannot be excluded. The use of a lag time model results in overestimation of the initial CL of ifosfamide compared to our model. Thus, an initial

CL of 3.48±0.88 L/h/m^2 was reported in 15 breast cancer patients after 5 g/m^2/24 h continuous i.v. infusion using a model with a lag time, compared to our findings of 1.77 L/h/m^2 (2.94 L/h) after 9 and 12 g/m^2/72 h continuous i.v. infusion.[2]

The observed interindividual variability in population parameters was small (less than 24.5%). For IC_{50}, the inclusion of interindividual variability did not improve the fit. This should not be interpreted as an absence of interindividual variability in this parameter, but only that the data did not contain sufficient information to estimate it. The proportional error of 13.6% and the additive error of 0.0763 µM described the residual variability not explained by the model. The residual variability may in part be explained by errors in the bioanalytical assay and errors in patient treatment (e.g. infusion duration) and sampling times.

In clinical studies with ifosfamide large variability has been observed in treatment effectiveness and toxicity.[12] Interpatient pharmacokinetic variability could be one of the factors that contributes to this variability in response. Our observations demonstrate that pharmacokinetic variability in our population was only modest. However, since the metabolites of ifosfamide are responsible for the treatment effectiveness and toxicity, interpatient pharmacokinetic variability of these compounds should also be characterized when studying dose response relationships. The increase in AUC of ifosfamide after doubling of the dose was only 56%. This emphasizes the importance of the integrating autoinduction into the modelling of non-linear ifosfamide pharmacokinetics.

In conclusion, the proposed model allowed quantification of the effect of autoinduction on the concentrations-time profiles of ifosfamide. The model demonstrated no necessity for a lag time in onset of induction. Although the small number of patients studied prevented the use of techniques such as data-splitting, cross-validation and bootstrapping for testing the predictive performance, the model was able to describe the individual pharmacokinetic profile of patients with adequate precision. Future utilization of this model may include investigating the relationship between the pharmacodynamics (e.g. bone marrow suppression, neurotoxicity) and the pharmacokinetics of ifosfamide. In order to achieve this plasma 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide metabolite data will have to be included in the model, since they are thought to be responsible for specific toxicities. Secondly, co-variables could also be studied for their possible predictiveness of the pharmacokinetics and pharmacodynamics of ifosfamide.

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Chapter 3.2

Population pharmacokinetics of ifosfamide

and its 2- and 3-dechloroethylated and

4-hydroxylated metabolites in resistant

small-cell lung cancer patients

Summary

This study aimed to develop a population pharmacokinetic model that could describe the pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxy-ifosfamide and calculate their plasma exposure and urinary excretion. Fourteen patients with small cell lung cancer received a 1-hour intravenous infusion of 1.0-3.0 g/m² ifosfamide for 1 or 2 days in combination with 175 mg/m² paclitaxel and carboplatin at AUC=6. A comparison was made between three population pharmacokinetic models without and with a time- or concentration-dependent development of autoinduction of ifosfamide. The latter model described the concentration-time profiles of ifosfamide best. Population values were estimated for clearance, volume of distribution, rate constant for enzyme degradation and the ifosfamide concentration at 50% of the maximum inhibition of the enzyme degradation at 2.49±0.47 L/h, 46.2±2.8 L, 0.114±0.040 h^{-1} and 21.4±2.6 µM, respectively. The Bayesian estimates of the pharmacokinetic parameters were used to evaluate the systemic exposure of ifosfamide and its metabolites in four ifosfamide schedules. Modest interindividual differences were observed in systemic exposure. Both ifosfamide and 4-hydroxyifosfamide exhibited a steeper dose-exposure relationship than the dechloroethylated metabolites. Dose-fractionation over two days compared to one day resulted in increased metabolite formation especially for 2-dechloroethylifosfamide, probably due to increased autoinduction. Renal recovery was only minor with 6.6% of the administered dose excreted unchanged and 9.8% as dechloroethylated metabolites. In conclusion, ifosfamide pharmacokinetics were best described by a model with ifosfamide concentration-dependent development of autoinduction. Population pharmacokinetics of the metabolites of ifosfamide and their exposures were estimated.

Introduction

Small cell lung cancer (SCLC) is highly sensitive to chemotherapy, but cure rates remain low with 5-year survival rates of 20-25%.[1] Responses may be followed by a rapid relapse within a few months and this usually goes along with development of tumour resistance against chemotherapy. Since salvage treatment rarely results in long-lasting disease control, most patients die from locally progressive or metastatic disease. A paclitaxel with carboplatin regimen has already proven activity with a tumour response rate of 73% in resistant SCLC.[2] Brain metastases are regularly observed in SCLC patients.[3] Therefore, ifosfamide, which is also active in SCLC was added to this regimen, because its activated metabolites are able to penetrate the blood brain barrier for 38-49% of the plasma concentrations.[4]

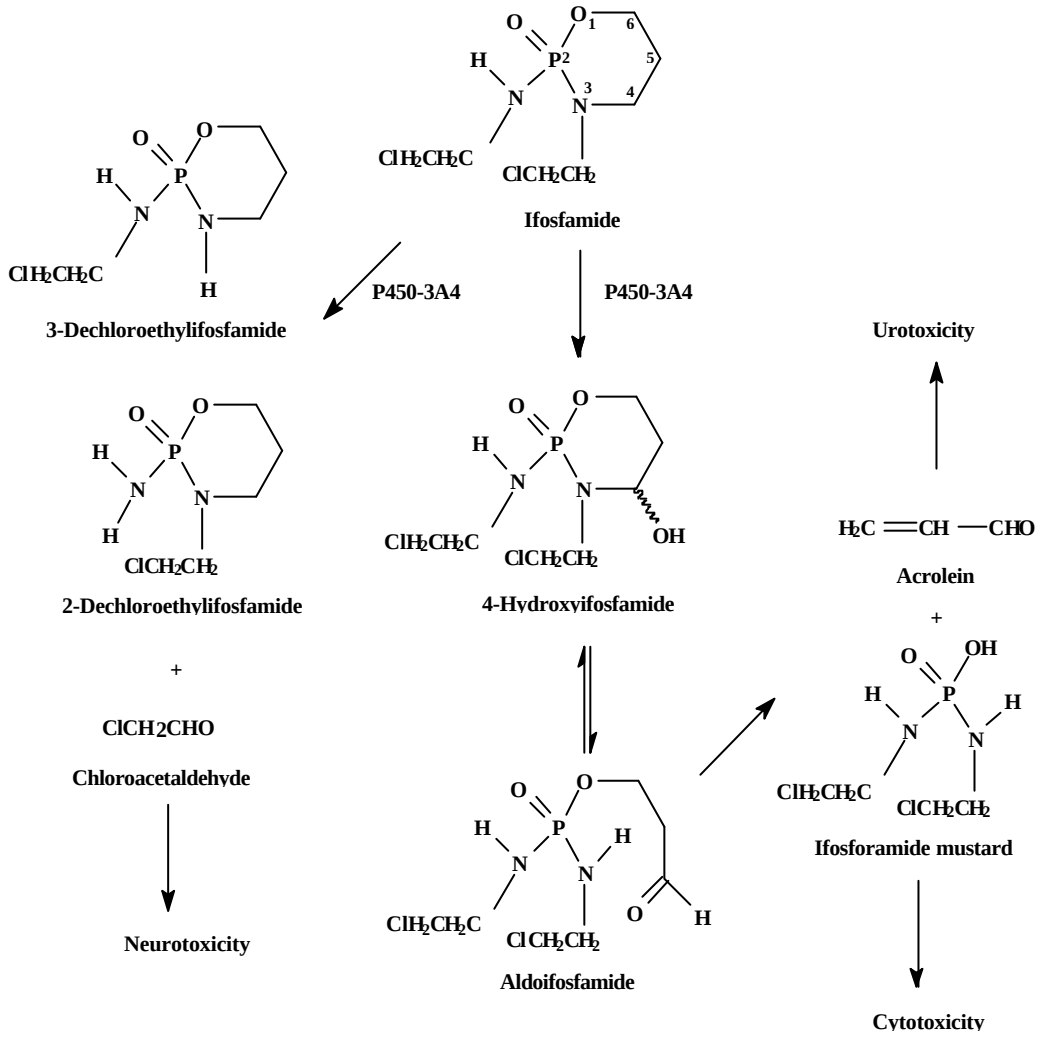

Figure 1. *Metabolism of ifosfamide.*

Ifosfamide (Holoxan®) is a prodrug, which needs activation by cytochrome P450-3A4 (CYP3A4) to 4-hydroxyifosfamide, as depicted in figure 1. Intracellular spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard.[5] Ifosfamide is deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide. Dechloroethylation yields an equimolar amount of chloroacetaldehyde, which was held responsible for neurotoxicity observed in about 10% of all patients receiving conven-

tional single-agent dosing of ifosfamide.[6] Activation is mainly conducted by CYP3A4 and deactivation by CYP3A4 and possibly CYP2B6.[7] Ifosfamide metabolism is subject to autoinduction, which will increase metabolism of ifosfamide in time. In order to evaluate ifosfamide pharmacokinetics in relation to pharmacodynamic outcome, the pharmacokinetics of metabolites emerging from both the activation and deactivation routes need to be included in the evaluation. Historically, systemic exposure, particularly the area under the plasma concentration-time profile (AUC), is used in oncology in the development of these relationships.[8-10] Large interpatient variability in clinical toxicity is observed in ifosfamide treatment.[11] This could possibly be explained by interpatient differences in ifosfamide pharmacokinetics due to autoinduction. A population pharmacokinetic approach allows assessment of this variability. In this study we aimed to compare the applicability and goodness-of-fit of three population pharmacokinetic models without or with a time-dependent or ifosfamide concentration-dependent development of autoinduction. The superior model will be applied to describe the ifosfamide concentration-time profiles of patients, who participated in a phase I study of the combination ifosfamide, paclitaxel and carboplatin. The Bayesian estimated pharmacokinetic parameters of ifosfamide were used to estimate the population pharmacokinetic parameters of the ifosfamide metabolites. The pharmacokinetics will be used to discuss ifosfamide and metabolite systemic exposure in four different ifosfamide treatment schedules.

Methods

Patients

Patients with resistant SCLC were hospitalized for administration of ifosfamide, paclitaxel and carboplatin in a phase I study. Inclusion criteria were: last chemotherapy given within 3 months, WHO performance status 2, white blood cell counts > 3 $x10^9$/L, platelets > 100 $x10^9$/L and normal renal and liver function tests. The study protocol was approved by the Ethics Board of the University Hospital Groningen. Written informed consent was obtained from each patient before entering the study protocol.

Drug administration

Ifosfamide (Holoxan®, ASTA Medica, Diemen) was delivered in vials with dry powder and was dissolved in distilled water and administered as an intravenous infusion in glucose 5%. Paclitaxel (Taxol®, Bristol-Myers Squibb, Woerden, The Netherlands) was delivered as a concentrated sterile solution, 6 mg/ml in 5 ml ampoules in polyoxyethylated castor oil (Cremophor EL) and dehydrated alcohol (1:1, v/v). Paclitaxel was diluted in a minimum volume of 250 ml and a maximum volume of 1000 ml of dextrose 5% or normal saline. Carboplatin (Paraplatin®, Bristol-Myers Squibb, Woerden, The Netherlands) was supplied as a lyophilized product that contained 150 mg carboplatin and 150 mg of mannitol as bulking agent. Immediately before use, the content of each vial was reconstituted with 15 ml water for injection and the total dose was added to 250 ml dextrose 5%. The dose of carboplatin in mg

was calculated according to the formula: dose (mg) = carboplatin clearance (ml/min) x AUC (min· mg/ml). Carboplatin clearance was calculated according to the Chatelut formula.[12]
Four different dose levels for ifosfamide were investigated in the phase I study. At the first dose level sampling was performed in four patients receiving ifosfamide 1,500 mg/m² on days 1 and 2 by means of a 1-hour intravenous infusion. At the second dose level four patients received ifosfamide 1,000 mg/m² on days 1 and 2. At the third dose level two patients received ifosfamide 2,000 mg/m² on day 1 by means of a 1-hour intravenous infusion. At the fourth dose level four patients received ifosfamide 3,000 mg/m² on day 1. At each dose level after the last ifosfamide administration, paclitaxel 175 mg/m² by means of a 3-hour intravenous infusion and carboplatin at AUC=6 min· mg/ml by means of a 30-minute intravenous infusion were administered.
To prevent bladder toxicity, mesna (Uromitexan®, ASTA Medica, Diemen) was administered in a total dose equal to the ifosfamide dose. Administration was partitioned equally over the i.v. infusion and twice orally three and seven hours, thereafter. To avoid acute allergic reactions all patients received dexamethasone 8 mg orally every 12 hours and 30 minutes before paclitaxel, clemastine 2 mg i.v. push 30 minutes before paclitaxel and ranitidine 50 mg i.v. push 30 minutes before paclitaxel. Anti-emetics used were ondansetron twice daily for the first three days and additionally dexamethasone and metoclopramide when needed.

Pharmacokinetic sampling

Pharmacokinetic sampling was performed during the first dosing cycle. The pharmacokinetic sampling at 0, 0.5, 1, 1.5, 2, 4, 8, 24, 24.5, 25, 25.5, 26, 28, 32 and 48 hours after start of the first ifosfamide infusion by means of an intravenous dwelling catheter was performed in patients receiving ifosfamide over two days (dose levels 1 and 2) and at 0, 1, 2, 5 and 24 hours in patients receiving ifosfamide for one day (dose levels 3 and 4). First 2 ml whole blood was drawn and discarded, followed by collection of 5 ml whole blood in a lithium heparin-coated Vacutainer® (Becton-Dickinson, Plymouth, UK) and placed on ice water. The plasma was immediately separated by centrifugation at 1,000 g for 5 min at 4°C. The plasma was aliquoted in three volumes of which two were precisely 1-ml volumes. To these 1-ml volumes, 100 µl 2 M semicarbazide solution at pH 7.40 were added to stabilize 4-hydroxyifosfamide. The remaining plasma was used for ifosfamide, 2- and 3-dechloroethylifosfamide analysis. Furthermore, urine was collected for up to 24 hours after the end of the last ifosfamide infusion and a representative sample of the collected urine was analysed for ifosfamide, 2- and 3-dechloroethylifosfamide. Both plasma and urine samples were stored at -20°C up to 1 month pending analysis.

Bioanalysis

Gas chromatography with selective nitrogen phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[13] Sample pre-treatment consisted of alkalinized liquid-liquid extraction with ethyl acetate, transfer of the organic extract, evaporation to dryness and subsequent reconstitution in ethyl acetate. This method proved to be specific, sensitive, accurate (93.3-104.5%) and precise (<5.5%) from

0.192 to 383 µM, with a lower limit of quantification (LLQ) of 0.192 µM for ifosfamide, 2- and 3-dechloroethylifosfamide.

High-performance liquid chromatography (HPLC) was used for determination of 4-hydroxyifosfamide pharmacokinetics.[14] In brief, this method determined the 4-hydroxyifosfamide-semicarbazone derivative in plasma. The HPLC column used was reversed phase C_8 with acetonitrile-0.025 M potassium dihydrogenphosphate (32:68 v/v) as mobile phase. Detection was performed at 230 nm. Sample pre-treatment consisted of liquid-liquid extraction with ethyl acetate. This method was specific, sensitive, accurate (94.1-107.9%) and precise (<7.2%) from 0.361 to 361 µM, with a LLQ of 0.361 µM 4-hydroxyifosfamide.

Data evaluation

Pharmacokinetic models were fitted to the data from l4 individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling).[15,16] NONMEM estimates population parameters as typical parameter values with corresponding interindividual variability, usually as its S.D., denoted as ω. This is accomplished by allowing each individual's data to be described by subject specific pharmacokinetic parameters P_i; this parameter is assumed to come from the distribution of parameters in the population according to equation 1.

$$P_i = P_{pop} \times \exp(\eta_i) \quad \text{(eq. 1)}$$

In which P_{pop} is the parameter value of a typical individual and η is the symmetrically distributed zero-mean variable with the S.D. denoted as ω. For mixed effects models the residual error corresponds to the difference between observed (C_{obs}) and predicted (C_{pred}) concentration by individual parameters (P_i). The residual or intraindividual error was described by a proportional and additive component according to equation 2,

$$C_{obs} = C_{pred} \times (1+\varepsilon_1) + \varepsilon_2 \quad \text{(eq. 2)}$$

in which ε_1 and ε_2 are zero-mean random variables with S.D.s σ_1 and σ_2. Either component of the residual error could be omitted if it did not provide an improvement of the fit of the data. Model accuracy was evaluated using goodness-of-fit plots and precision of the parameters estimates. For the graphical goodness-of-fit analysis extensive plotting was available through the use of X-pose, a purpose-built set of subroutines in S-PLUS (Mathsoft, 1997).[17] In comparison between models the objective function value (which was minus two times the log-likelihood) provided by NONMEM was used. For hierarchical models the difference in objective function value is approximately χ-squared distributed and formal testing between models can be performed using the log-likelihood ratio test.[15] For non-hierarchical models the objective function value cannot be used for formal testing, but we considered a difference of 9 units between models of the same number of parameters and identical databases to present a real difference in description of the data.[18] Three models (model 1-3) for the description of the plasma concentration-time profiles of ifosfamide were compared. Furthermore, a model describing the pharmacokinetics of the metabolites was developed.

Model 1

Model 1 is a one-compartment model describing the change of the amount of ifosfamide (A_{ifo}, µmol) in the central compartment over time by equation 3,

$$\mathbf{t < T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = R - \left(CL \times A_{ifo} / V_{ifo}\right)$$

$$\mathbf{t > T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = -\left(CL \times A_{ifo} / V_{ifo}\right) \qquad \text{(eq. 3)}$$

in which T_{inf} (h) is the infusion duration, R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL (L· h^{-1}) the ifosfamide clearance and V_{ifo} (L) the volume of distribution. The concentration of ifosfamide C_{ifo} (µM) is the ratio of A_{ifo} and V_{ifo}. Thus in model 1, CL is a constant parameter.

Model 2

In model 2, CL has been replaced by a clearance of ifosfamide which changes over time ($CL_{(t)}$, L· h^{-1}), thus yielding equation 4.

$$\mathbf{t < T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = R - \left(CL_{(t)} \times A_{ifo} / V_{ifo}\right)$$

$$\mathbf{t > T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = -\left(CL_{(t)} \times A_{ifo} / V_{ifo}\right) \qquad \text{(eq. 4)}$$

In turn, $CL_{(t)}$ can be described by defining a hypothetical enzyme compartment. $CL_{(t)}$ can then be described by an initial clearance (CL_{init}, L· h^{-1}) multiplied by the relative amount of enzyme (A_{enz}) in the hypothetical enzyme compartment as given in equation 5.

$$CL_{(t)} = CL_{init} \times A_{enz} \qquad \text{(eq. 5)}$$

The change of A_{enz} over time in the enzyme compartment is dependent on C_{ifo} as follows,

$$\frac{dA_{enz}}{dt} = K_{enz,in} - K_{enz,out} \times A_{enz} \times \left(1 - \frac{C_{ifo}}{C_{ifo} + IC_{50}}\right) \qquad \text{(eq. 6)}$$

in which $K_{enz,in}$ (mol· h^{-1}) is the zero-order rate constant for enzyme formation/activation, $K_{enz,out}$ (h^{-1}) is the first-order rate constant for enzyme degradation/inactivation and IC_{50} (µM) is the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation. Before treatment with ifosfamide (t=0) steady-state levels of the enzyme were assumed. Hence, the change in enzyme amount was zero. $K_{enz,in}$ was equal to $K_{enz,out}$ times the amount of enzyme at t=0 ($A_{enz,\ t=0}$). By scaling $A_{enz,\ t=0}$ to a value of 1 (without a dimension), $K_{enz,in}$ could be substituted for $K_{enz,out}$ yielding equation 7.

$$\frac{dA_{enz}}{dt} = K_{enz,out} - K_{enz,out} \times A_{enz} \times \left(1 - \frac{C_{ifo}}{C_{ifo} + IC_{50}}\right) \qquad \text{(eq. 7)}$$

In which A_{enz} is the relative amount of enzyme (no dimension). The induction half-life of the enzyme ($T_{½\ enz}$, h) was calculated by the ratio of ln(2) and $K_{enz,out}$.[19]

Model 3

In model 3 a concentration independent increase of $CL_{(t)}$ over time during steady-state infusions was used. Boddy et al. described a similar model but with an exponential increase after a lag time from an initial clearance to a maximum clearance.[20] This model described

$CL_{(t)}$ with a time dependent increase from CL_{init} as described by equation 8.

$$CL_{(t)} = CL_{ini} \times (1 + K_{enz} \times t) \quad \text{(eq. 8)}$$

In which K_{enz} ($L \cdot h^{-2}$) was the enzyme induction rate constant and t (h) the time.

Model 4

After obtaining the individual pharmacokinetic parameters $CL_{(t)}$ and V_{ifo} from the superior model, these parameters were used in the modelling of the metabolite pharmacokinetics. The change in the amount of a metabolite (A_m) over time could be described by equation 9.

$$\frac{dA_m}{dt} = \left(\frac{F_m}{V_m} \times CL_{(t)} \times \frac{A_{ifo}}{V_{ifo}} \right) - (K_m \times A_m) \quad \text{(eq. 9)}$$

In which K_m (h^{-1}) is the elimination rate constant of the metabolite and F_m is the fraction of ifosfamide metabolized to the metabolite. The values for F_m and the volume of distribution of the metabolite (V_m, L) cannot be estimated separately in this model. Therefore, the ratio of F_m over V_m was estimated: F^* (L^{-1}). The ratio was used to calculate the area under the concentration-time curve (AUC_m) of the metabolite, as described by equation 10.

$$AUC_m = \frac{D}{K_m \times \frac{V_m}{F_m}} \quad \text{(eq. 10)}$$

In which D (mol) was the ifosfamide dose administered to the patient. The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model according to equation 1. The residual or intraindividual variability of ifosfamide and metabolites kinetics were described separately with a proportional and additive term according to equation 2. The Bayesian estimates of the areas under the concentration-time curves (AUC) of ifosfamide and its metabolites were obtained by describing the cumulative concentrations of these compounds over a given time. AUCs were obtained with extrapolation of ifosfamide and metabolite concentration-time profiles to 144 hours after the start of the infusion, where concentrations were below the LLQ. The urinary excretions (UE) for ifosfamide and 2- and 3-dechloroethylifosfamide were calculated as the equimolar amount of the administered ifosfamide dose recovered in urine over 96 hours.

Results

Fourteen patients were evaluable for plasma pharmacokinetics and ten patients for urine recovery measurements. The pharmacokinetic data-base consisted of 122 ifosfamide, 120 2-dechloroethylifosfamide, 122 3-dechloroethylifosfamide and 117 4-hydroxy-ifosfamide plasma concentrations. The observed ifosfamide and metabolite concentration-time profiles are shown in figure 2. The pharmacokinetic parameters for ifosfamide and a comparison of the three population pharmacokinetic models are represented in table 1.

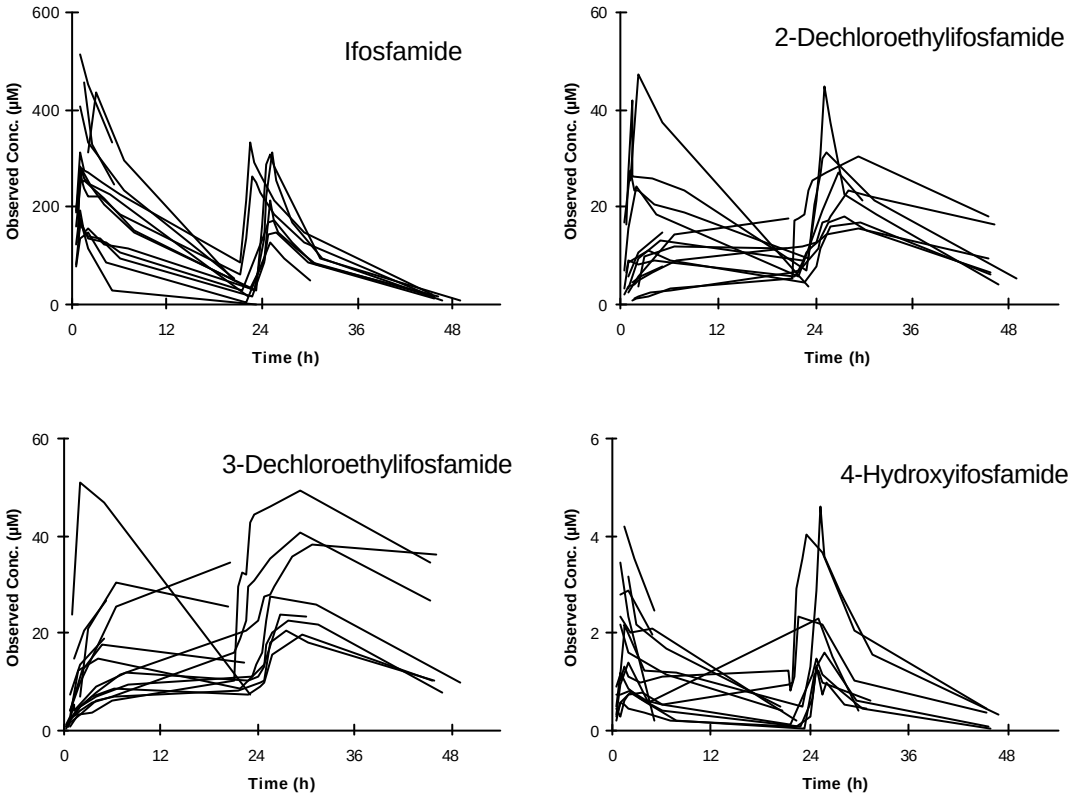

Figure 2. *Observed concentrations of ifosfamide and metabolites in 14 patients treated with four different schedules of ifosfamide, followed by paclitaxel and carboplatin.*

Table 1. *Estimates of pharmacokinetic population parameters for ifosfamide (IFO) with their relative standard error, interindividual variability and residual variability by model 1, 2 and 3.*

Parameter	Mean estimate	Relative error	Interindividual variability
MODEL 1 obj f=975			
CL (L· h^{-1})	5.30	22%	29%
V_{ifo} (L)	42.4	3%	17%
P.E. IFO	13.9%		
A.E. IFO (µM)	16.2		
MODEL 2 obj f=934			
CL_{init} (L· h^{-1})	2.49	19%	41%
V_{ifo} (L)	46.2	6%	17%
$K_{enz,out}$ (h^{-1})	0.114	35%	-
$T_{½,enz}$(h)	6.1	-	-
IC_{50} (µM)	21.4	12%	-
P.E. IFO	17.4%		
A.E. IFO (µM)	-		
MODEL 3 obj f=954			
CL_{init} (L· h^{-1})	3.39	26%	30%
V_{ifo} (L)	46.9	6%	19%
K_{enz} (h^{-1})	0.0269	38%	-
$T_{½,enz}$(h)	25.8		
P.E. IFO	14.9%		

A.E. IFO (µM)	10.5

Obj f=objective function, CL=ifosfamide clearance, V_{ifo}=volume of distribution of ifosfamide, P.E.=proportional intraindividual error, A.E.=additive intraindividual error, $CL_{ini,ifo}$=initial CL, $K_{enz,out}$=first-order rate constant for enzyme degradation, $T_{½,enz}$=induction half-life of the enzyme, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, K_{enz}=zero-order rate constant for enzyme degradation.

The goodness-of-fit of a model was described by its objective function. The objective functions of model 1 and 3 were 41 and 20 points, respectively, higher than that of model 2. We therefore conclude that model 2 is the most appropriate model to describe the observed data. The residual error represents the remaining variability not explained by the structural model; it is described by an additive and proportional component. Model 1 has the lowest proportional error, but with the highest additional error. On the other hand, model 2 only required a proportional error. Overall, model 2 had the lowest residual variability with 17.4%. The relative error in the estimate of a population parameter is a measure of precision of estimation. These precisions in all three models for ifosfamide were less than 38% and considered good. All three models estimated V_{ifo} similar. V_{ifo} was estimated between 42.4 and 46.9 L, with an interindividual variability of 17-19%. CL_{init}s of model 2 and 3 were also similar with 2.49±0.47 and 3.39±0.88 $L \cdot h^{-1}$ and an interindividual variability of 41 and 30%, respectively. For the parameters describing the increase in $CL_{(t)}$, such as $K_{enz,out}$, IC_{50} and K_{enz}, inclusion of interindividual variability did not improve the fit. This should not be interpreted as an absence of interindividual variability in these parameters, but only that the data do not contain sufficient information to estimate these. The $CL_{(t)}$ as function of time and calculated by model 2 for each patient is shown in figure 3.

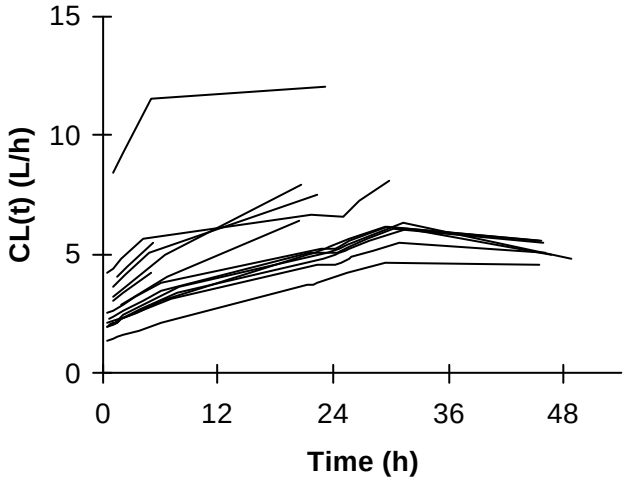

Figure 3. *Individual ifosfamide clearance-time ($CL_{(t)}$) profiles in 14 patients with an ifosfamide concentration-dependent increase of ifosfamide clearance (model 2). The profiles were obtained on basis of the individual Bayesian parameter estimates provided by NONMEM.*

The population pharmacokinetic parameters of the metabolites are given in table 2. The model predictions and individual Bayesian predictions versus observed ifosfamide and metabolite concentrations with their distribution around the line of identity are shown in figure 4. Accuracy of the estimates was reasonable (relative errors ranged from 14 to 66%). Modest

interindividual variability was observed in the population parameters (29 to 52%). In contrast to 3-dechloroethylifosfamide and 4-hydroxyifosfamide, 2-dechloroethyl-ifosfamide was best estimated by a residual error model with only an additive error.

Table 2. *Estimates of pharmacokinetic population parameters for 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) with their relative standard error, interindividual variability and residual variability based upon the Bayesian parameter estimates of model 2.*

Parameter	Mean estimate	Relative error	Interindividual variability
F^*_{2DCE} (L^{-1})	0.0426	40%	52%
K_{2DCE} (h^{-1})	2.22	36%	-
F^*_{3DCE} (L^{-1})	0.00771	14%	36%
K_{3DCE} (h^{-1})	0.138	16%	47%
F^*_{4OHIF} (L^{-1})	0.0180	60%	-
K_{4OHIF} (h^{-1})	9.90	66%	29%
P.E. 2DCE	-		
A.E. 2DCE (µM)	6.89		
P.E. 3DCE	33.1%		
A.E. 3DCE (µM)	0.366		
P.E. 4OHIF	30.5%		
A.E. 4OHIF (µM)	0.218		

F^*=ratio of the fraction of ifosfamide metabolized to the metabolite and the volume of distribution of the metabolite, K=first-order elimination rate constant of metabolite, P.E.=proportional residual error, A.E.=additive residual error.

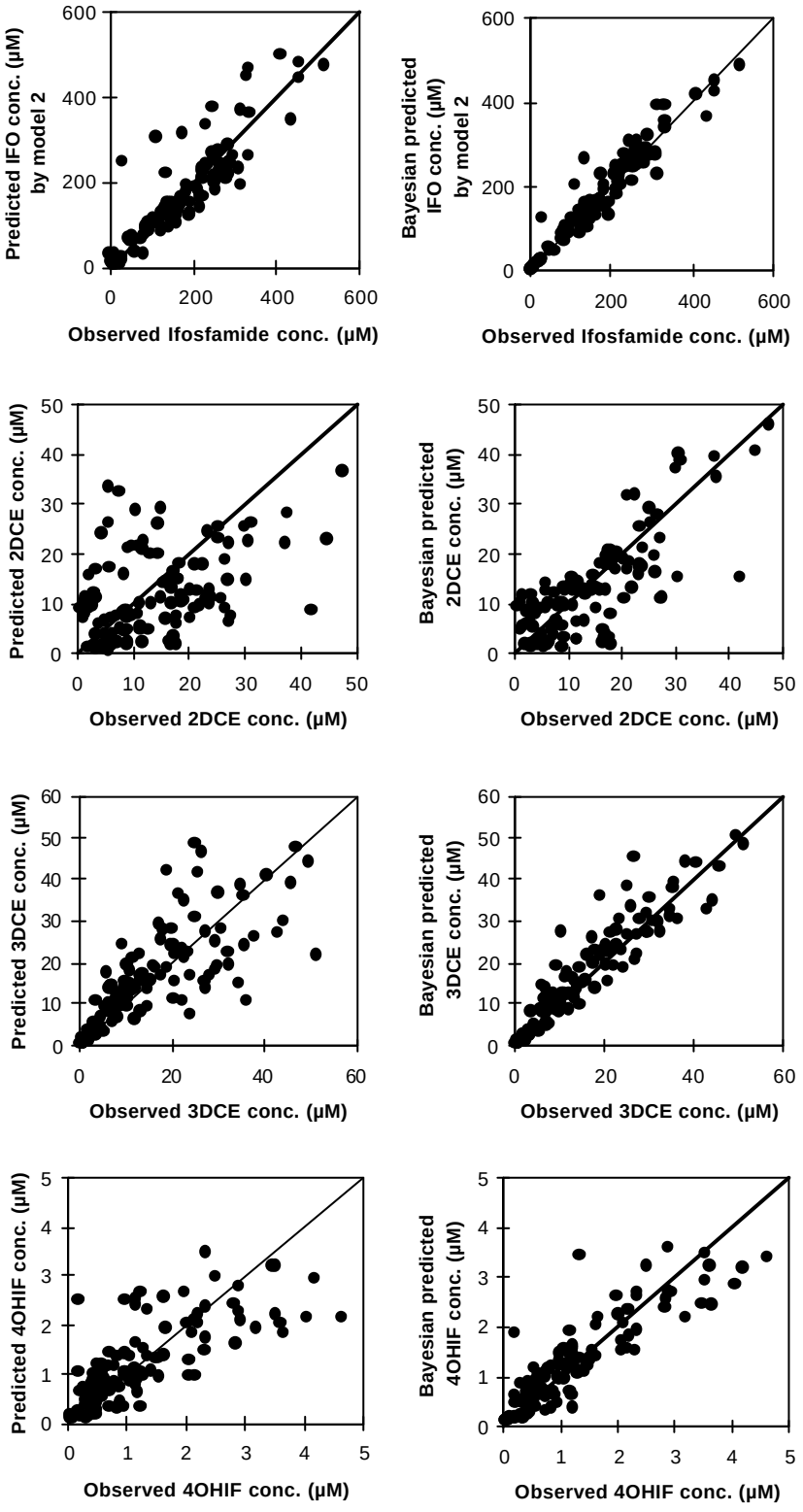

Figure 4. *The relationships between model predictions, individual Bayesian estimates and observed ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) concentrations.*

The pharmacokinetic parameter estimates for the metabolites were used to estimate the AUCs. Figure 5 depicts the relationship between the systemic exposures of ifosfamide and metabolites and the absolute dose. Linearity was observed in the relationship of the absolute ifosfamide dose and AUC with ifosfamide ($p<0.001$) and 4-hydroxyifosfamide ($p=0.003$), but not 2-dechloroethylifosfamide ($p=0.458$) and 3-dechloroethylifosfamide ($p=0.065$). The urinary excretion of unchanged ifosfamide and its dechloroethylated metabolites are represented in table 3. The relationships between the urinary excretion of ifosfamide ($p=0.238$) and the dechloroethylated metabolites ($p=0.015$), and the absolute ifosfamide doses are represented in figure 6. Considerable interindividual variability is observed in the urinary recoveries.

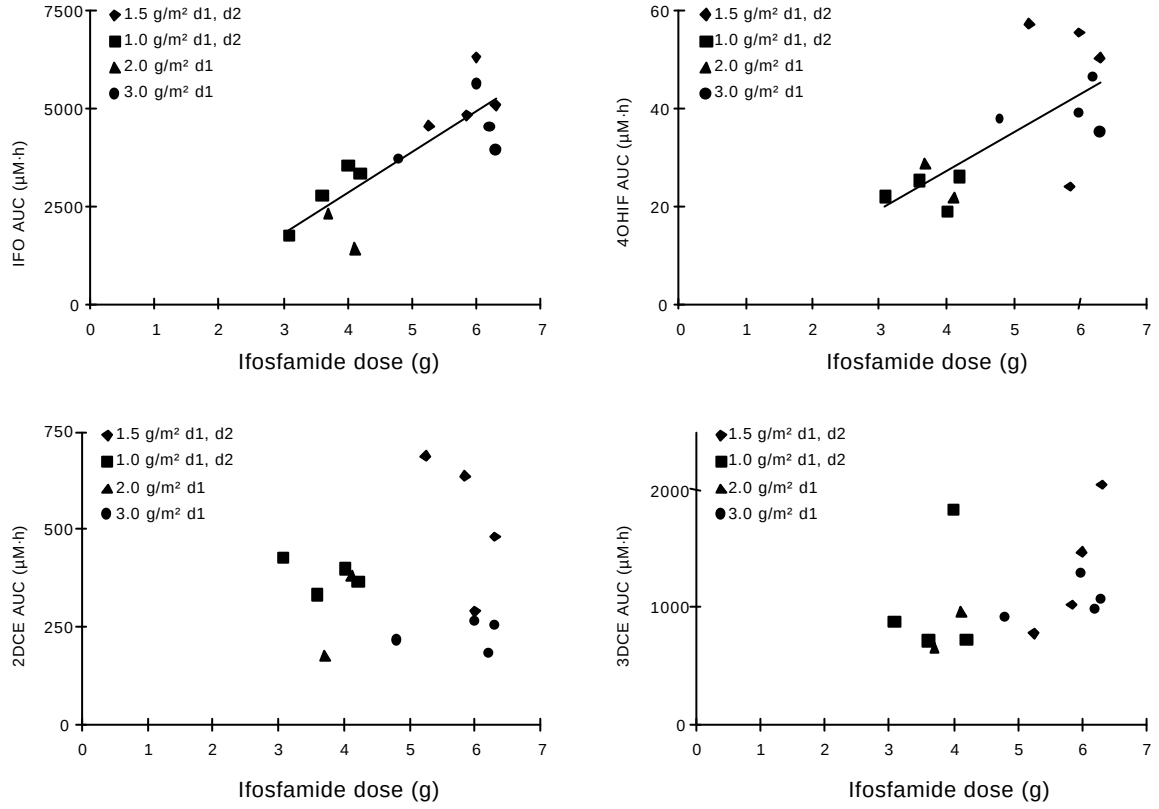

Figure 5. *The relationship between the systemic exposure of ifosfamide (IFO) and 2-dechloroethyl-ifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) after four different dosing schedules in 14 patients. Systemic exposure was based on the area under the plasma concentration-time curve (AUC).*

Table 3. *Percentage of dose excreted in urine either unchanged as ifosfamide (IFO) or as metabolite 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide (3DCE) in 10 patients.*

Patient	IFO Dose (g)	IFO (%)	2DCE (%)	3DCE (%)	Total (%)
1	6.30	4.0	1.6	6.0	11.6
2	6.00	3.9	1.6	5.4	10.9
3	5.25	6.3	1.4	4.4	12.1
4	5.85	7.8	1.7	4.2	13.7
5	4.00	2.8	1.3	6.9	11.0
6	3.60	13.6	3.6	7.5	24.7
7	4.20	9.8	3.4	7.9	21.1
8	3.08	4.6	7.6	15.4	27.6
13	6.00	5.1	2.8	4.9	12.8
14	6.20	8.4	2.4	8.4	19.2
Mean±SD		6.6±3.3	2.7±1.9	7.1±3.3	16.5±6.2

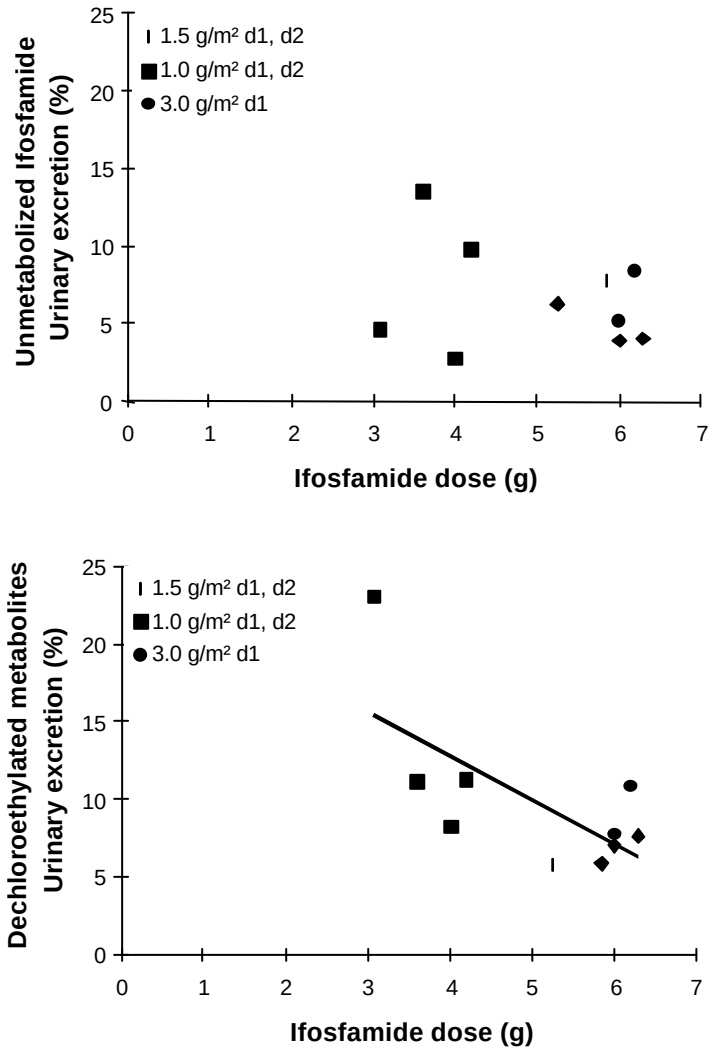

Figure 6. *The relationship between the urinary recovery of ifosfamide and the dechloroethylated metabolites and the absolute ifosfamide dose in 10 patients.*

Discussion

Three different population models were compared to describe pharmacokinetics of ifosfamide in patients treated with the combination ifosfamide, paclitaxel and carboplatin. The Bayesian estimates of the ifosfamide parameters provided by the superior model were used in the modelling of the metabolite pharmacokinetics. All population models for ifosfamide were based on one-compartment models. Introduction of a peripheral compartment did not significantly increase the goodness-of-fit of the model. Urinary recovery of unchanged ifosfamide and its dechloroethylated metabolites was minor. As a result, splitting the ifosfamide clearance into an inducible metabolic clearance and a non-inducible renal clearance also did not improve the goodness-of-fit of the model.

Model 3 was based on a model by Boddy et al. describing autoinduction by an exponential increase of the initial clearance to a maximum value.[20] This model was developed on a population of patients receiving a 72-hours continuous infusion schedule. The application of this model in our patient population resulted in a linear increase of clearance over time from 0 to 48 hours, thus a more simple linear increase with time could be used without estimation of the maximum value of clearance.

Model 2 described autoinduction of ifosfamide best using a dynamic hypothetical enzyme compartment. This enzyme amount could be increased by inhibition of enzyme degradation and/or inactivation by ifosfamide. Ifosfamide reduces protein synthesis.[21] Ifosfamide could, therefore, also reduce catalytic enzymes responsible for CYP degradation and/or inactivation, resulting in an increased ifosfamide clearance. An increased amount of enzyme could also be achieved by an increase in synthesis of the enzyme itself. Such a model has been proposed for cyclophosphamide, an isomer of ifosfamide.[22] Whatever the physiological mechanism, all will result in an increased amount of enzyme and both modelling approaches will yield the same result.[22]

The ifosfamide concentration dependent autoinduction model (2) describes an increase in the clearance of ifosfamide during the first 24 hours with an observed maximum on the second day followed by a decrease. In one patient an increased CL_{init} was observed. This patient metabolized ifosfamide more rapidly than other patients, but without an altered rate of autoinduction, which was reflected in elevated levels of dechloroethylated and hydroxylated metabolites. Although the ifosfamide and metabolite concentrations were outlyers compared to other patients, the population approach with estimation of interindividual variability still provided an adequate Bayesian pharmacokinetic description of this patient.

The metabolite model adequately described the pharmacokinetics of the metabolites. The fraction of the ifosfamide dose converted to a metabolite (F_m) was less than one for all metabolites. Thus according to equation 9, V_m of all metabolites was always smaller than V_{ifo}. This is in accordance with the expectation that the metabolism of an exogenous compound like ifosfamide will lead to more hydrophilic compounds.

Pharmacokinetics of 4-hydroxyifosfamide are formation rate-limited as reflected in the high value for K_{4OHIF}. Therefore, elimination of 4-hydroxyifosfamide could also be estimated by fixing the K_{4OHIF} to 100 times the elimination of ifosfamide. Nevertheless, fixing of K_{4OHIF} did

not improve the objective function significantly. Therefore, it was decided to estimate K_{4OHIF}, although the imprecision of the estimation was relatively high.

Both ifosfamide and 4-hydroxyifosfamide exhibited a more steep dose-exposure relationship than dechloroethylated metabolites, as represented in figure 5. The lack of a distinct dose-exposure relationship of the dechloroethylated metabolites could be explained by their relatively high interindividual differences in exposure. Although the four different treatment schedules of ifosfamide administration did not result in a wide dose range, some trends in the exposures due to dose-fractionating could be observed. The AUC of 3 g/m² ifosfamide given over one or two days did not differ significantly. However, administration over two days resulted in relatively higher metabolite formation. The AUC of 2-dechloroethylifosfamide, particularly, was increased after administration of ifosfamide over two subsequent days. This can be explained by autoinduction resulting in increased metabolite formation.

Renal excretion of unchanged ifosfamide was 6.6±3.3% and of the dechloroethylated metabolites 9.8±5.1% of the administered dose. The urinary excretions are in accordance with previously reported data in patients receiving fractionated dosing of single agent ifosfamide.[5] Previous stability studies indicated no degradation of ifosfamide, 2- and 3-dechloroethylifosfamide during the 24-hour collection interval.[13]

The relationship between the ifosfamide dose and the percentage excreted as dechloroethylated metabolites (figure 6) were largely determined by patient no. 8 (table 3). Markedly, in this patient the second lowest dose-corrected exposure to ifosfamide was observed in combination with the highest and second highest dose-corrected exposure to 2- and 3-dechloroethylifosfamide, respectively. This was also reflected in the clearance values (approximately 8 L/h) of this patient.

In conclusion, ifosfamide concentration-time profiles were best described by modelling the development of autoinduction with an ifosfamide concentration-dependent increase in ifosfamide clearance. Systemic exposures to ifosfamide and metabolites were calculated using Bayesian estimated pharmacokinetic parameters.

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Chapter 3.3

Influence of infusion duration on the pharmacokinetics of ifosfamide and metabolites

Summary

The anticancer drug ifosfamide is a prodrug requiring activation through 4-hydroxyifosfamide to ifosforamide mustard to exert cytotoxicity. Deactivation of ifosfamide leads to 2- and 3-dechloroethylifosfamide and the release of potentially neurotoxic chloroacetaldehyde. The aim of this study was to quantify and to compare the pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard in short (1-4 h), medium (24-72 h) and long infusion durations (96-240 h) of ifosfamide. An integrated population pharmacokinetic model was used to describe the autoinducible pharmacokinetics of ifosfamide and its four main metabolites in 56 patients. The rate by which autoinduction of the metabolism of ifosfamide developed and the fractions metabolized to 2- and 3-dechloroethylifosfamide were found to be significantly dependent on the infusion schedule. The observed differences in the parameters were, however, comparable to their interindividual variability and were, therefore, considered to be of minor clinical importance. Autoinduction caused a less than proportional increase in the area under the ifosfamide plasma concentration-time curve (AUC) and more than proportional increase in metabolite exposure with increasing ifosfamide dose. During long infusion durations dose-corrected exposures (AUC/D) were significantly decreased for ifosfamide and increased for 3-dechloroethylifosfamide when compared with short infusion durations. No differences in dose-normalized exposure to ifosfamide and metabolites were observed between short and medium infusion durations. This study demonstrates that the duration of ifosfamide infusion influences the exposure to the parent and its metabolite 3-dechloroethylifosfamide. The observed dose and infusion duration dependency should be taken into account when the pharmacodynamics of different infusion schedules are evaluated.

Introduction

Ifosfamide (Holoxan®, Ifex®) is an alkylating agent which has been proven to be active against a number of solid tumours and haematological malignancies in adults and children. Currently, ifosfamide is mostly used in combination with other anticancer drugs, but also as single agent. Many different infusion schedules are in use, e.g. continuous infusion over either 24, 72 or 240 hours with doses of 5, 9 or 7.5-15 g/m² ifosfamide, respectively.[1,2]

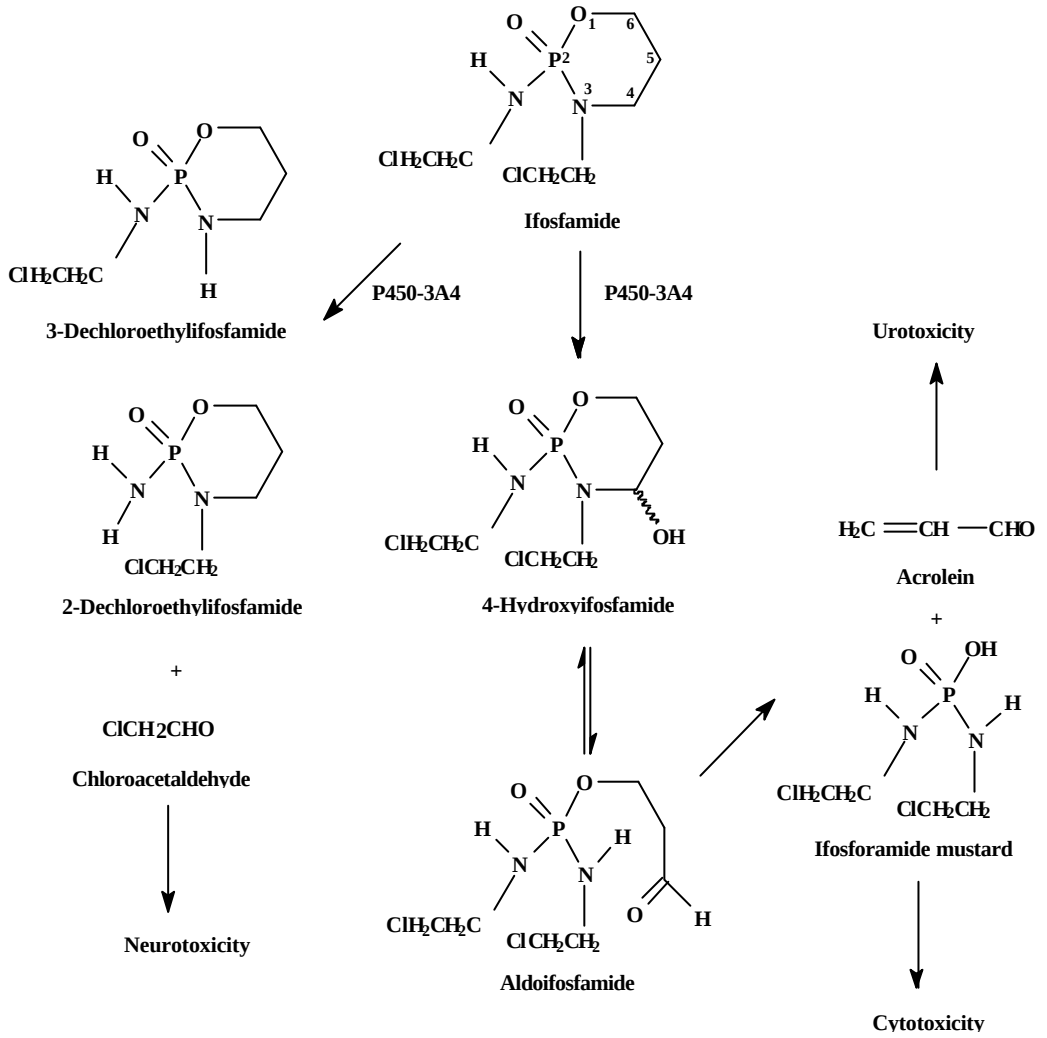

Figure 1. *Metabolism of ifosfamide.*

Ifosfamide is a prodrug, which needs activation by cytochrome P450-3A4 (CYP3A4) to 4-hydroxyifosfamide as depicted in figure 1. Spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard and acrolein.[3] The alkylating activity of ifosforamide mustard is responsible for both the anti-tumour activity and haematological toxicity. Acrolein causes haemorrhagic cystitis, which can generally be prevented by mesna co-administration. Ifosfamide is deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide. Each dechloroethylation reaction yields an equimolar amount of chloroacetaldehyde, which is held responsible for neurotoxicity observed in about 10% of all patients receiving conventional single-agent dosing of ifosfamide.[4] In addition, observed renal tubular abnormalities may also be correlated with the formation of chloroacetaldehyde.[3] Chloroacetaldehyde is very unstable. Therefore,

assessment of 2- and 3-dechloroethylifosfamide exposure is preferred when quantifying the relationship between the pharmacokinetics and neurotoxicity after ifosfamide infusion. Ifosfamide is subject to autoinduction leading to an increase in metabolism of ifosfamide over time.[5] Several comparative studies observed that fractionation of the ifosfamide dose leads to increased autoinduction.[3] Pharmacokinetic studies can provide further insight into the effect of the infusion schedule on the autoinduction of ifosfamide metabolism. Aim of this study is to quantify and compare the pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard in short (1-4 h), medium (24-72 h) and long infusion durations (96-240 h) of ifosfamide. Assessment of pharmacokinetics will indicate whether the development of autoinduction is dependent on the dose and/or infusion duration and may further aid in the design of ifosfamide infusion schedules.

Methods

Eligibility criteria

Thirty-three patients received ifosfamide (Holoxan®, ASTA Medica, Diemen, The Netherlands) as intravenous (i.v.) infusion as part of the regular therapy with ifosfamide as single agent or as combination chemotherapy with cisplatin, carboplatin, etoposide, adriamycine, vincristine or methotrexate, during the period of March 1997 to April 2000. Typical infusion schedules used for ifosfamide were 1.2 g/m² once daily over 1, 3 or 4 hours for three consecutive days, 1.5 g/m² once daily in 22.5 hours for two consecutive days, 5 g/m² in 24 hours once and 9 g/m² by means of a 72-hours continuous infusion. The study protocol was approved by the Ethics Board of the Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute. Another group of 23 patients received a 10-day continuous infusion of single-agent ifosfamide using an ambulatory pump. This group was treated at the Leiden University Medical Center, in a study that was approved by the hospital Ethics Board.[2] Supportive care consisted of mesna and extensive hydration to prevent haemorrhagic cystitis and bicarbonate to prevent acidosis. Standard 5-HT_3 blockers as anti-emetics and methylene blue (a neurotoxicity antidote) were given when indicated.[4] Pharmacokinetic sampling was performed after written informed consent was obtained from the patients.

Pharmacokinetic sampling

Blood samples were drawn before the start of, during, and after the end of the infusion at selected time points, depending on the infusion schedule. Ten ml of whole blood was collected in a lithium heparin-coated Vacutainer® (Becton-Dickinson, Plymouth, UK) and placed on ice water. The plasma was immediately separated by centrifugation at 1,000 g for 5 min at 4°C. The plasma was aliquoted in four volumes of which three were precisely 1-ml volumes. 100 µl 2 M semicarbazide solution pH 7.4 were added to two 1-ml volumes to stabilize 4-hydroxyifosfamide. 100 µl 1 M sodium chloride, 2 M semicarbazide solution pH 8.0 were added to another 1-ml volume to prevent 4-hydroxyifosfamide degradation to ifosforamide mustard and to stabilize the latter. The remaining plasma was used for

ifosfamide, 2- and 3-dechloroethylifosfamide analysis. Urine was collected from the start of the (first) infusion until 24 hours after the end of the (last) ifosfamide infusion and was analysed for ifosfamide, 2- and 3-dechloroethylifosfamide. Both plasma and urine samples were stored at -70°C pending analysis.

Bioanalysis

Gas chromatography with selective nitrogen-phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[6] Sample pre-treatment consisted of alkalinized liquid-liquid extraction with ethyl acetate, transfer of the organic extract to another tube, evaporation to dryness and subsequent reconstitution in ethyl acetate. This method was validated and proved to be specific, sensitive, accurate (93.3-104.5%) and precise (within and between-day <5.5%) within the concentration range of 0.192 to 383 µM, with a lower limit of quantification (LLQ) of 0.192 µM for ifosfamide, 2- and 3-dechloroethylifosfamide. High-performance liquid chromatography (HPLC) was used for determination of 4-hydroxyifosfamide plasma levels.[7] In brief, this method determined the 4-hydroxyifosfamide-semicarbazone derivative in plasma. Sample pre-treatment consisted of liquid-liquid extraction with ethyl acetate. The HPLC column used was reversed phase C_8 with acetonitrile-0.025 M potassium dihydrogenphosphate (32:68 v/v) as mobile phase. Detection was performed at 230 nm. This method was specific, sensitive, accurate (94.1-107.9%) and precise (within and between-day <7.2%) in the concentration range of 0.361 to 361 µM, with a LLQ of 0.361 µM 4-hydroxyifosfamide (measured as semicarbazone derivative). Plasma concentrations of ifosforamide mustard were determined using a validated HPLC method.[8] In brief, this method determined the ifosforamide mustard-diethyldithio-carbamate derivative in plasma. Sample pre-treatment consisted of derivatization followed by liquid-liquid extraction with acetonitrile. Derivatization was achieved by incubating 500 µl plasma with 350 µl 1 M sodium chloride 0.1 M potassium dihydrogenphosphate buffer pH 8.0 and 50 µl diethyldithiocarbamate for 30 min at 70°C. The HPLC column used was a reversed phase C_8 column with acetonitrile-0.025 M potassium dihydrogenphosphate pH 8.0 (32:68 v/v) as mobile phase. Detection was performed at 276 nm. This method was specific, sensitive, accurate (101.4-110.0%) and precise (<15.8%) in the concentration range of 0.452 to 226 µM, with a LLQ of 0.452 µM ifosforamide mustard (measured as diethyldithiocarbamate derivative).

Data evaluation

Pharmacokinetic models were fitted to the plasma concentration data from the individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling, version V 1.1, double precision, first order estimation).[9,10] The population pharmacokinetic models resulted in model predictions and Bayesian individual predictions for the concentrations of ifosfamide and metabolites. The non-linear pharmacokinetics of ifosfamide were described using a recently developed model, which incorporated the development of autoinduction.[5] According to this recent study the time dependent pharmacokinetics of ifosfamide can be described by a one-compartment model (figure 2).

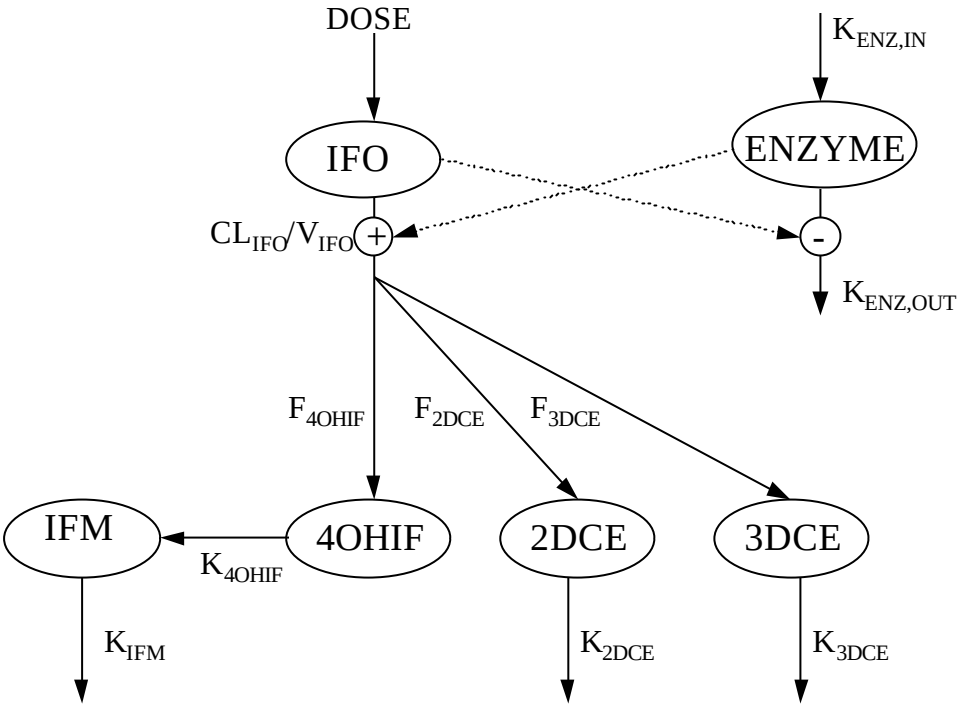

Figure 2. *Pharmacokinetic model for ifosfamide metabolism describing the autoinducible pharmacokinetics of ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM). Autoinduction is modelled with a hypothetical enzyme compartment described by an enzyme formation rate ($K_{enz,in}$) and elimination rate ($K_{enz,out}$). The amount of enzyme increases the clearance of IFO (CL_{IFO}). The amount of IFO, described by the IFO concentration and IFO volume of distribution (V_{IFO}), inhibits $K_{enz,out}$. Metabolite compartments are described by metabolite formation (F) and elimination rates (K).*

The change of the amount of ifosfamide (A_{ifo}, µmol) in the central compartment over time is described by equation 1,

$$\mathbf{t < T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = R - \left(CL \times A_{ifo} / V_{ifo}\right) \text{ and}$$

$$\mathbf{t > T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = -\left(CL \times A_{ifo} / V_{ifo}\right) \qquad \text{(eq. 1)}$$

in which T_{inf} (h) is the infusion duration, R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL (L· h^{-1}) the ifosfamide clearance, which changes over time and V_{ifo} (L) the volume of distribution. The predicted concentration of ifosfamide C_{ifo} (µM) is the ratio of A_{ifo} and V_{ifo}. The development of autoinduction is described using a hypothetical enzyme compartment. CL is expressed as initial clearance ($CL_{ini,ifo}$, L· h^{-1}) multiplied by the relative amount of enzyme (A_{enz}) in the hypothetical enzyme compartment, as given in equation 2.

$$CL = CL_{ini,ifo} \times A_{enz} \qquad \text{(eq. 2)}$$

The change of A_{enz} (a relative measure with no dimension) over time in the enzyme compartment is dependent on C_{ifo} as follows,

$$\frac{dA_{enz}}{dt} = K_{enz,out} - K_{enz,out} \times A_{enz} \times \left(1 - \frac{C_{ifo}}{C_{ifo} + IC_{50}}\right) \qquad \text{(eq. 3)}$$

in which $K_{enz,out}$ (h^{-1}) is the first-order rate constant for enzyme degradation/inactivation and IC_{50} (µM) is the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation. At t=0 and A_{enz}=1, the enzyme formation rate ($K_{enz,in}$) is equal to $K_{enz,out}$ (figure 2). The induction half-life of the enzyme ($T_{½enz}$, h) was calculated by the ratio of ln(2) and $K_{enz,out}$.

The pharmacokinetics of ifosfamide and metabolites were described sequentially: Bayesian estimations (posthocs) of the pharmacokinetic parameters of ifosfamide were used during the assessment of the pharmacokinetics of the metabolites. The change in the amount of a metabolite (A_m) over time for 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide could be described by equation 4,

$$\frac{dA_m}{dt} = \left(F_m \times CL \times \frac{A_{ifo}}{V_{ifo}}\right) - \left(K_m \times A_m\right) \qquad \text{(eq. 4)}$$

in which K_m (h^{-1}) is the elimination rate constant of the metabolite and F_m is the fraction of ifosfamide metabolized to the metabolite. The values for F_m and the volume of distribution of the metabolite (V_m, L) cannot be estimated separately in this model. Therefore, the ratio of F_m over V_m was estimated: F^* (L^{-1}). The change in the amount of ifosforamide mustard (A_{ifm}) over time could be described by equation 5,

$$\frac{dA_{ifm}}{dt} = \left(F_{ifm} \times K_{4ohif} \times A_{4ohif}\right) - \left(K_{ifm} \times A_{ifm}\right) \qquad \text{(eq. 5)}$$

in which K_{ifm} (h^{-1}) is the elimination rate constant of ifosforamide mustard and F_{ifm} is the fraction of 4-hydroxyifosfamide metabolized to ifosforamide mustard. Equation 5 can be rearranged to equation 6,

$$\frac{dA_{ifm}}{dt} = \left(F_{ifm} \times V_{4ohif} \times K_{4ohif} \times C_{4ohif}\right) - \left(K_{ifm} \times A_{ifm}\right) \qquad \text{(eq. 6)}$$

in which V_{4ohif} (L) is the volume of distribution of 4-hydroxyifosfamide and C_{4ohif} (µM) is the 4-hydroxyifosfamide concentration. The values for F_{ifm} and the volumes of distribution of 4-hydroxyifosfamide and ifosforamide mustard (V_{ifm}, L) cannot be estimated separately in this model and were, therefore, replaced by one parameter: F^{**}_{ifm}, which has no dimension.

The residual or intraindividual variability of the pharmacokinetics of ifosfamide and metabolites were described separately with a combined proportional and additive term. The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model.

The posthocs of the areas under the concentration-time curves (AUC) of ifosfamide and its metabolites were obtained by describing the cumulative concentrations of these compounds over a given time. AUCs were obtained with extrapolation of ifosfamide and metabolite concentration-time profiles up to 72 hours after the end of the (last) ifosfamide infusion, where concentrations were below the LLQ. The effect of the infusion duration on the autoinduction of ifosfamide metabolism and its effect on the metabolite exposures was investigated by calculating dose-corrected AUCs. The dose-corrected AUC was obtained by dividing the AUC by the administered ifosfamide dose. A non-parametric test was conducted to determine significant differences in dose-corrected AUCs between the infusion schedules. The pharmacokinetics of each group of infusion schedules were compared with the other two groups using the Kolmogorov-Smirnov test for two independent samples. The influence of the independent variable infusion duration was tested on the estimation of each population

parameter of ifosfamide, 2- and 3-dechloroethylifosfamide according to equation 7,

$$P_{pop} = \theta_1^{short} \times \theta_2^{medium} \times \theta_3^{long} \quad \text{(eq. 7)}$$

in which P_{pop} is a population parameter, θ_1 is the typical value of that parameter with short duration infusions, θ_2 is the change in θ_1 with medium infusion duration, θ_3 is the change in θ_1 with long infusion duration. If the 95% confidence interval (95%-CI) of the estimate of θ_2 and θ_3 incorporated 1, no significant difference between the short infusion durations with medium or long infusion durations was observed. The urinary excretions (UE) for ifosfamide and 2- and 3-dechloroethylifosfamide were calculated as the equimolar amount of the administered dose recovered in urine. Differences between the urinary excretions of the different infusion durations were tested for significance using the non-parametric Mann-Whitney test. Statistical analysis was performed using SPSS software (Version 6.1 for Windows; SPSS, Inc., Chicago, IL, USA). The level of significance (p) was set at 0.05. All tests for significance were two-tailed.

Results

A total of 56 patients was divided into three sub-populations with 12 patients receiving short (1-4 h), 21 patients receiving medium (24-72 h) and 23 patients receiving long (96-240 h) infusion durations of ifosfamide (table 1). The number of patients in which 4-hydroxyifosfamide and ifosforamide mustard were analysed were insufficient for the separate estimation of the pharmacokinetics of these metabolites for each infusion schedule. The pharmacokinetic population parameters with their standard error and interindividual variability and the residual variability of ifosfamide and 2- and 3-dechloroethylifosfamide are presented in table 2.

Table 1. *Number of patients pharmacokinetically assessed for ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) for each infusion schedule.*

	All infusions		Short (1-4 h)		Medium (24-72 h)		Long (96-240 h)	
	patients n	samples n	patients n	samples n	patients n	samples n	patients n	samples n
IFO	56	598	12	143	21	184	23	271
2DCE	53	502	12	141	21	180	20	181
3DCE	53	513	12	140	21	181	20	192
4OHIF	15	135	2	27	13	108	0	0
IFM	8	70	2	20	6	50	0	0

Table 2. *Estimates of population pharmacokinetic parameters of ifosfamide (IFO) 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide (3DCE) with their relative standard error of the mean, interindividual variability and residual variability.*

Parameter	Mean	RE	IIV	Parameter	Mean	RE	IIV
$CL_{ini,ifo}$ (L/h)	3.01	7%	26%	F^*_{2DCE} (L^{-1})	0.0161	24%	43%
V_{ifo} (L)	45.9	10%	28%	θ^{medium}	0.487	34%	

$K_{enz,out}$ (h^{-1})	0.0815	13%	22%	K_{2DCE} (h^{-1})	0.385	19%	39%
θ^{long}	0.48	39%		$P.E._{.2DCE}$	1.73		
$T_{½enz}$ (h)	8.5			$A.E._{.2DCE}$ (µM)	20.6%		
IC_{50} (µM)	24.4	33%	55%	F^*_{3DCE} (L^{-1})	0.0109	20%	35%
$P.E._{.IFO}$	2.84			θ^{medium}	0.666	23%	
$A.E._{.IFO}$ (µM)	12.6			K_{3DCE} (h^{-1})	0.124	18%	19%
				$P.E._{.3DCE}$	3.31		
				$A.E._{.3DCE}$ (µM)	17.0%		

RE=Relative error of the mean, IIV=interindividual variability, $CL_{ini,ifo}$=initial ifosfamide clearance, V_{ifo}=volume of distribution of ifosfamide, $K_{enz,out}$=autoinduction formation rate, θ=difference in parameter estimation due to long or medium infusion duration, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, P.E.=proportional intraindividual error, A.E.=additive intraindividual error, F^*=ratio of fraction metabolized and volume of distribution of metabolite, K=first-order rate constant for metabolite elimination.

Table 3. *Estimates of pharmacokinetic population parameters of 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) with their relative standard error of the mean, interindividual variability and residual variability.*

4OHIF				IFM			
Parameter	Mean	RE	IIV	Parameter	Mean	RE	IIV
F^*_{4OHIF} (L^{-1})	0.126	3%	30%	F^{**}_{IFM}	0.514	16%	
K_{4OHIF} (h^{-1})	73.8	3%	8%	K_{IFM} (h^{-1})	3.63	35%	73%
$P.E._{.4OHIF}$	22.8%			$P.E._{.IFM}$	50.2%		
$A.E._{.4OHIF}$ (µM)	0.300			$A.E._{.IFM}$ (µM)	-		

RE=Relative error of the mean, IIV=interindividual variability, F^*=ratio of fraction metabolized and volume of distribution of metabolite, K=first-order rate constant for metabolite elimination, F^{**}_{IFM}=fraction metabolized to IFM, P.E.=proportional intraindividual error, A.E.=additive intraindividual error.

The corresponding estimates of 4-hydroxyifosfamide and ifosforamide mustard are listed in table 3. The estimates of most pharmacokinetic parameters were not influenced by the duration of infusion. Only minor differences were found for $K_{enz,out}$, F^* of 2-dechloroethylifosfamide (F^*_{2dce}) and 3-dechloro-ethylifosfamide (F^*_{3dce}). $K_{enz,out}$ during long infusion durations was 52% lower in comparison with short and medium infusion durations. F^*_{2dce} and F^*_{3dce} decreased with 51% and 33%, respectively, after medium infusion durations when comparing to short and long infusion durations. No outliers or trends could be observed in the plots of the population predictions and individual Bayesian predictions of the concentrations versus the observed concentrations (plots not shown). The observed and individual Bayesian estimated pharmacokinetic profiles of ifosfamide of all patients are presented in figure 3. The urinary excretion of unchanged ifosfamide and its dechloroethylated metabolites are presented in table 4.

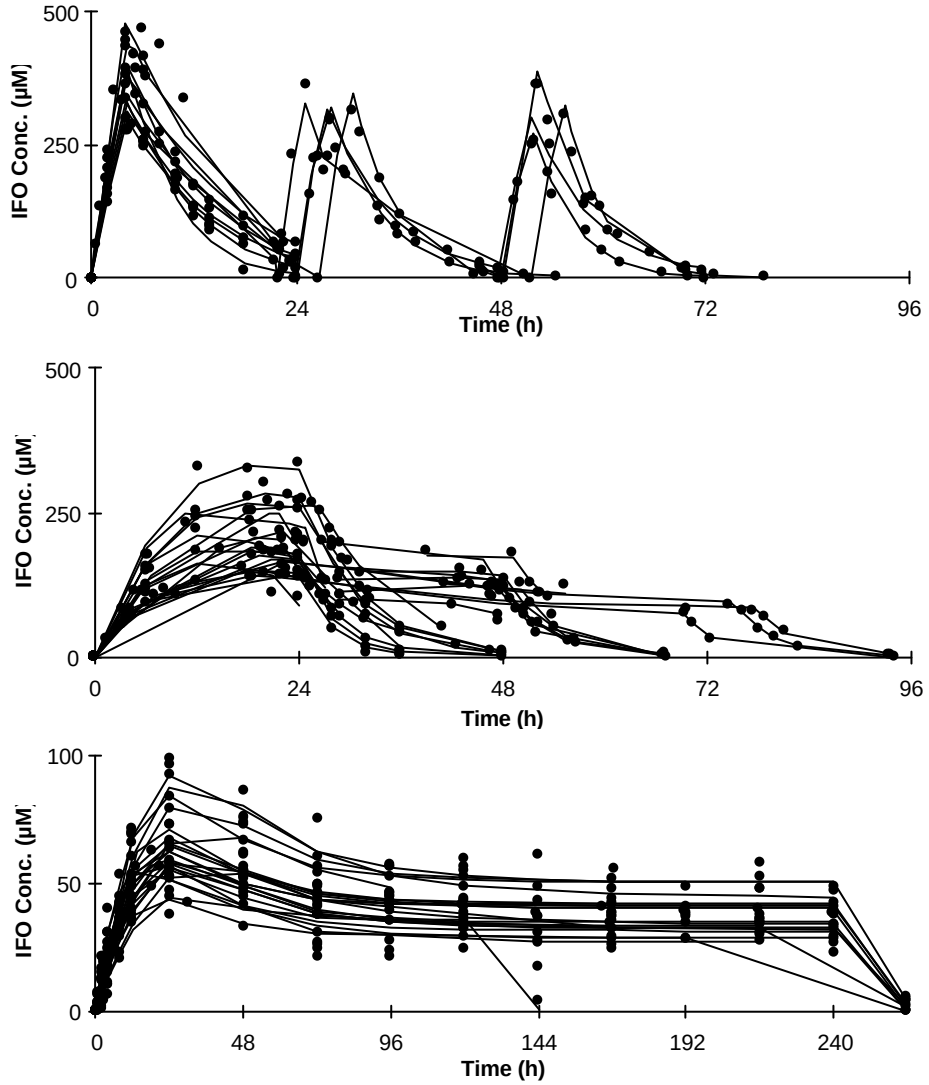

Figure 3. *The Bayesian individual predicted plasma concentration-time profiles of ifosfamide (IFO) for all patients during short (1-4 h), medium (24-72 h) and long (96-240 h) infusion durations. The observations are represented by the dots.*

Table 4. *Percentage of dose excreted in urine either unchanged as ifosfamide (IFO) or as metabolite 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide (3DCE) in 40 patients.*

Infusion schedule	n	IFO (%) Mean±SD*	2DCE (%) Mean±SD	3DCE (%) Mean±SD	Sum (%) Mean±SD*
short (1-4 h)	3	21.2±6.6	5.6±2.0	14.9±5.4	41.7±10.2
medium (24-72 h)	14	14.7±7.2	7.1±3.6	11.6±5.0	33.4±14.7
long (96-240 h)	23	8.6±2.6	2.9±2.6**	11.4±2.6	22.9±3.4

SD=Standard Deviation, *significantly different for all infusion durations, **significantly different from medium infusion durations.

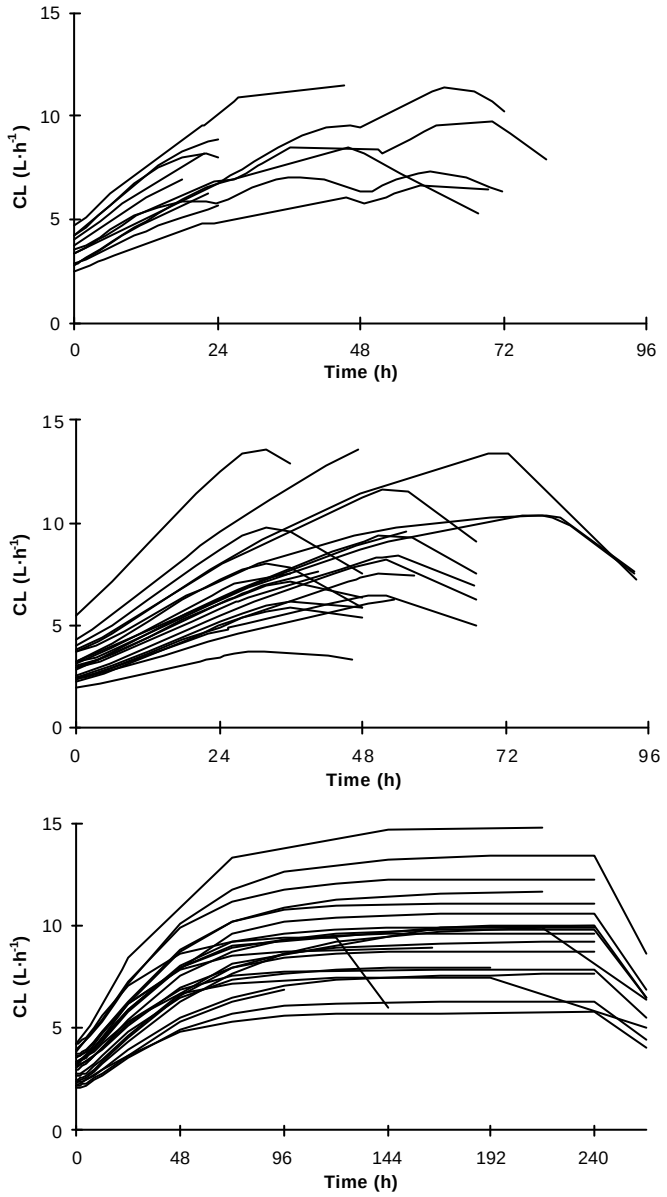

Figure 4. *Individual time profiles of the clearance of ifosfamide (IFO) for all patients during short (1-4 h), medium (24-72 h) and long (96-240 h) infusion durations. The profiles were obtained from the individual Bayesian parameter estimates based on the total population.*

Increase of infusion duration resulted in a significant decrease of urinary recovery of unchanged ifosfamide and the sum of ifosfamide and metabolites. For ifosfamide values for short and long infusion durations were 21.2±6.6% and 8.6±2.6%, respectively. No clear trend was observed for 2- and 3-dechloroethyl-ifosfamide, although urine recovery of 2-dechloroethylifosfamide during long infusions was slightly lower than during medium infusion duration. The individual profiles of the ifosfamide clearances over time of each infusion schedule are depicted in figure 4. Modest interindividual variability was observed. The clearance of ifosfamide increased during the infusion and decreased after cessation. Notably, during the first 24 hours for all patients the model predicted an average doubling of the clearance.

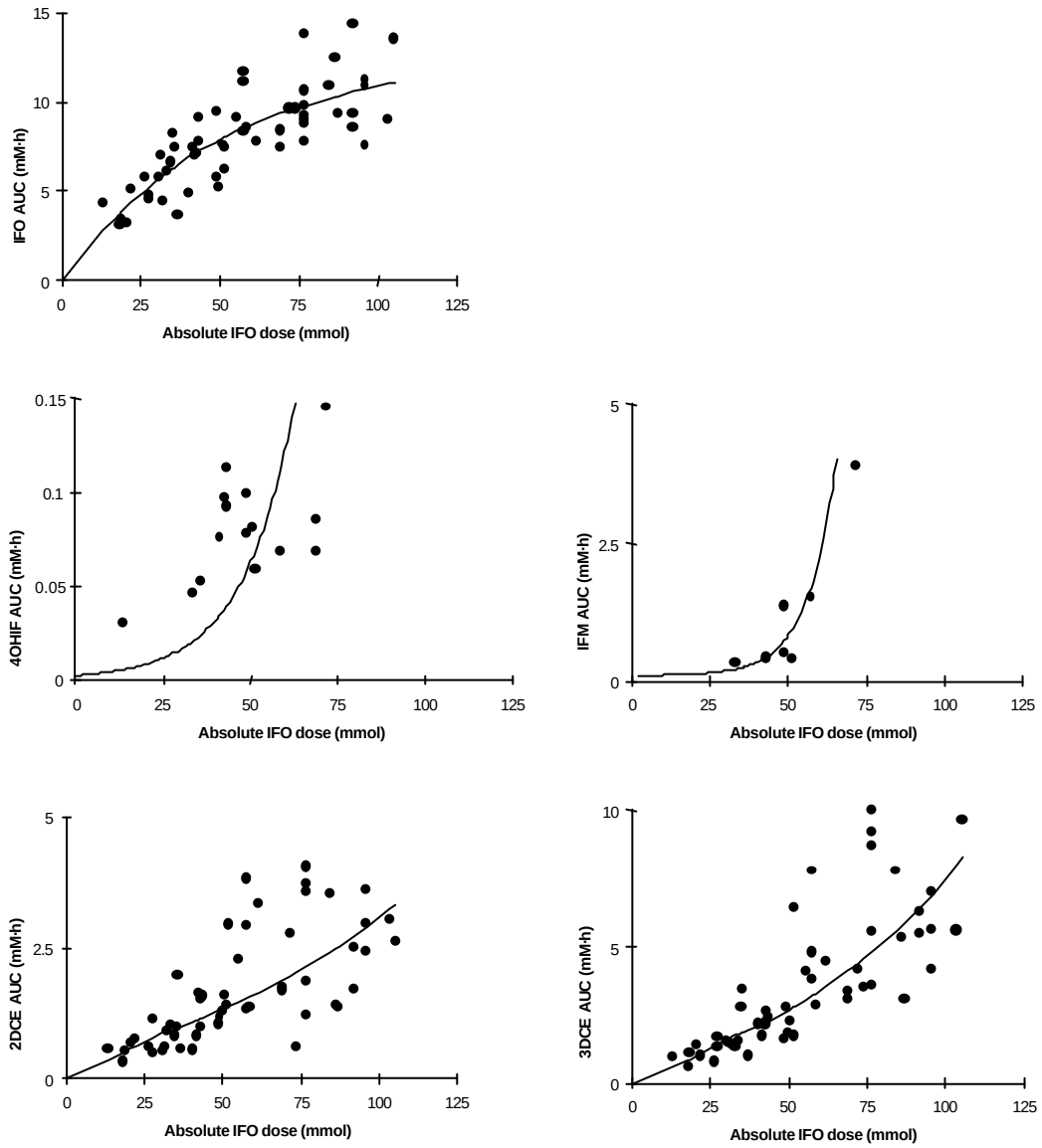

Figure 5. *The relationships (with Loess-smooths) between the ifosfamide dose (mmol) and ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) exposures as calculated by the area under the concentration-time curve (AUC) of each patient.*

Figure 5 depicts the relationships between the AUCs of ifosfamide and its metabolites and the ifosfamide dose for the total population. The AUC of ifosfamide increased less than proportional with increase in absolute ifosfamide dose. In contrast, the AUCs of the metabolites increased more than proportional with increase in absolute ifosfamide dose.

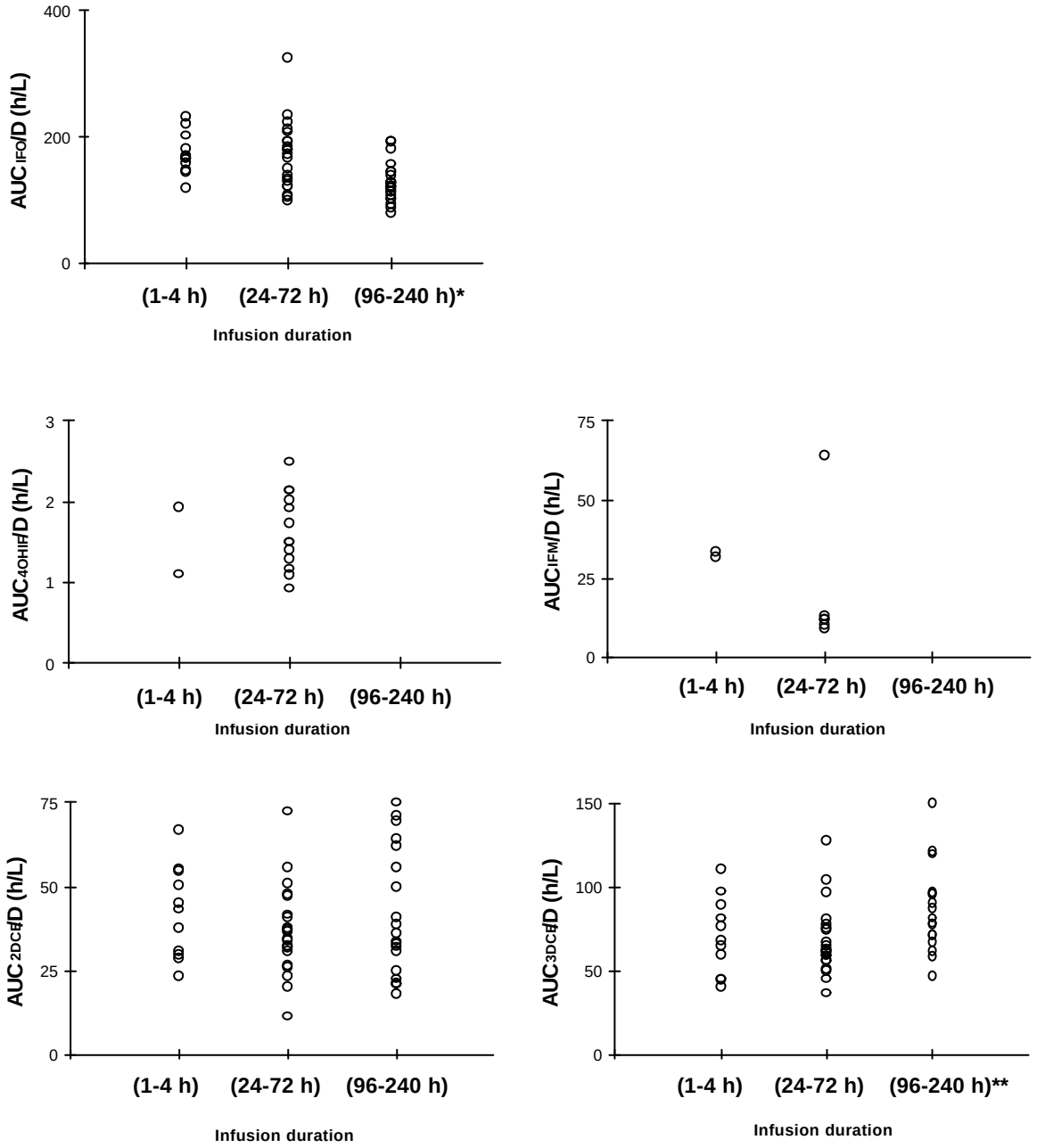

Figure 6. *The relationship between the ifosfamide infusion duration (h) and ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) dose-standardized exposures as calculated by the ratio of the area under the concentration-time curve (AUC) and the ifosfamide dose of each patient. *Significantly decreased 26% (p<0.001) and 25% (p<0.05), respectively, compared to 1-4 h and 24-72 h infusions. **Significantly increased 45% (p<0.01) compared to 24-72 h infusions.*

The dose-corrected AUCs are depicted in figure 6. The dose-corrected exposure to ifosfamide during long infusions was decreased 26% (p<0.001) and 25% (p<0.05) when compared to short and medium infusion duration, respectively. In comparison with medium infusion durations dose-corrected exposure to 3-dechloroethylifosfamide during long infusion

durations was increased with 45% ($p<0.01$).

Discussion

This study investigated the effect of the duration of the ifosfamide infusion on the pharmacokinetics of ifosfamide and its metabolites. In a review on the clinical pharmacokinetics of ifosfamide in 1994 it was proposed that fractionation of the ifosfamide dose increased the metabolism of ifosfamide.[11] However, more recent studies have indicated that the ifosfamide infusion schedule does not influence the degree of metabolism. In a comparative study in paediatric patients receiving 3 g/m² ifosfamide administered as a 1-hour infusion for three consecutive days or 9 g/m² ifosfamide administered as a 72-hour continuous infusion, no major differences in exposure were observed except a minor decrease in exposure to deactivated metabolites with the bolus infusion.[12] In addition, no differences in metabolic exposures were found in a similar study in adults.[13] Singer and co-workers concluded that there is no identifiable pharmacokinetic basis for insistence on either bolus or infusional methods of ifosfamide administration.

In our study a group of 56 patients was divided into three sub-populations receiving either short (1-4 h), medium (24-72 h) or long (96-240 h) infusion durations with ifosfamide. All patients studied received ifosfamide as part of standard therapy for the treatment of various malignancies. A previously developed autoinduction model was applied to assess the population pharmacokinetics of ifosfamide and metabolites.[5,14] This model describes the development of autoinduction with a relationship between the ifosfamide plasma concentration and a hypothetical, dynamic enzyme compartment, which is responsible for ifosfamide metabolism. Thus, clearance of ifosfamide over time changes with ifosfamide concentrations. From some of the patients included in the study only a limited number of blood samples was available for pharmacokinetic analysis. In such cases conventional non-compartmental analysis of the concentration-time profiles does not allow accurate estimation of the pharmacokinetic parameters due to insufficient data. The applied population approach with Bayesian estimation on the other hand does not have these drawbacks.

Statistically significant differences were found for $K_{enz,out}$ between long and short infusion durations and for F^*_{2dce} and F^*_{3dce} between medium and short infusion durations. These differences in parameter estimates were considered to be minor (33%-52%) and not clinically relevant, since they were in the same order of magnitude as the interindividual variability of the respective parameters (22%-43%). The observed differences may be explained by different amounts of data within the concentration-time profiles of the various infusion schedules, which are necessary for the estimation of these pharmacokinetic parameters. Estimation of $K_{enz,out}$ in long infusion durations is more accurate than in short infusion durations, because the effect of autoinduction is more pronounced. The relatively lower number of samples drawn during the first 24 hours of medium infusion durations, compared to short infusion durations, may have resulted in different estimations of the formation rates of the deactivated metabolites. All other estimates of the population parameters were not significantly influenced by infusion duration. This indicates that no clinically relevant differences were observed in the pharmacokinetics between the different infusion durations.

The population pharmacokinetic parameters were in accordance with a recent study on the

pharmacokinetics of ifosfamide and metabolites after administration of 9-12 g/m² ifosfamide in a 72-hour continuous intravenous infusion.[15] However, in the referred study values for F^*_{3dce} and K_{3dce} were 0.075±0.097 L^{-1} and 1.5±2.0 h^{-1}, respectively, which is approximately tenfold higher than observed in the present study (0.011±0.002 L^{-1} and 0.12±0.02 h^{-1}, respectively, table 2). The difference may be explained by the lower accuracy of the estimates in the former study. Interestingly, K values of the metabolites are greater than the value calculated for ifosfamide ($CL_{ini,ifo}/V_{ifo}$). This indicates that the observed decay in plasma of the metabolites is formation rate limited.

Table 4 indicates that considerable amounts of unchanged ifosfamide are excreted in the urine. Separate estimation of renal clearance, however, did not improve the goodness-of-fit of the model. The urinary excretion data found in this study were similar to previous observations. Gilard et al. reported urinary excretion of 18, 16 and 7% for ifosfamide, 2- and 3-dechloroethylifosfamide, respectively, in patients receiving 3g/m²/3 hours ifosfamide for 3 days.[16] The decrease in excretion of ifosfamide with increasing infusion duration can be explained by the development of the autoinduction. The decrease in excretion of ifosfamide with increasing infusion durations can be explained by decreasing AUCs due to autoinduction. For 2- and 3-dechloroethylifosfamide increased AUCs would theoretically produce increased urine recovery. This was however not observed. The mechanism of this discrepancy remains to be elucidated. Nevertheless, the latter observation is in accordance with the previously reported finding that the urinary excretions of 2- and 3-dechloroethylifosfamide were superimposable for the different infusion schedules in paediatric patients.[17]

The AUC of ifosfamide increased less than proportional with the absolute dose and the AUCs of the metabolites increased more than proportional with the absolute ifosfamide dose. This can be explained by a greater extent of autoinduction due to higher ifosfamide plasma concentrations (equations 2, 3 and 4). These findings are in accordance with a previous report in which the pharmacokinetic model for ifosfamide was developed.[5] The dose-corrected exposures to ifosfamide and its metabolites changed with increasing infusion duration. In comparison with short infusions, a decrease of 25% in dose-corrected ifosfamide exposure and an increase of 45% in 3-dechloroethylifosfamide exposure were observed with long infusion durations. The decrease in ifosfamide exposure can be explained by an increased average clearance during the long infusions (figure 4).

As a result of the increased average clearance of ifosfamide during long infusions dose corrected exposure to the dechloroethylated metabolites were expected to be increased. A significant increase was observed for 3-dechloroethylifosfamide. For 2-dechloroethylifosfamide an increase of dose corrected exposure may be obscured by the large interindividual variability (figure 6). Unfortunately, the effect of infusion duration on the dose corrected exposure to 4-hydroxyifosfamide and ifosforamide mustard could not be evaluated since no samples were available for the long infusions. Nevertheless, the observed increased exposure to the dechloroethylated metabolites during long infusion durations demonstrates the importance of the assessment of the pharmacokinetics when comparing the pharmacodynamics during different infusion durations.

In conclusion, a population pharmacokinetic model for ifosfamide and its metabolites was used to investigate any differences in ifosfamide metabolism due to the duration of the ifosfamide infusion. Estimates of $K_{enz,out}$, F^*_{2dce} and F^*_{3dce} were found to depend on the

infusion schedule. The clinical relevancy of the observed differences was however considered to be of minor importance. Concentration dependent development of autoinduction produced a less than proportional increase in ifosfamide exposure and more than proportional increase in metabolite exposure with increasing ifosfamide dose. Schedule dependency could be demonstrated for the dose-corrected exposures to ifosfamide and 3-dechloroethylifosfamide between short and long infusion durations, which could be explained by the increased average clearance during infusion. No change in dose-corrected exposure to ifosfamide and metabolites was observed between short and medium infusion durations in this population. The observed dependency on dose and infusion duration should be taken into account when the pharmacodynamics of different infusion schedules of ifosfamide are evaluated.

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Chapter 4.1

Population pharmacokinetics and pharmacodynamics of ifosfamide and metabolites after a 72-hour continuous infusion in patients with soft tissue sarcoma

Summary

The population pharmacokinetics and pharmacodynamics of the cytostatic agent ifosfamide and its main metabolites 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were assessed in patients with soft tissue sarcoma. Patients received 9 or 12 g/m² ifosfamide administered as a 72-hour continuous intravenous infusion. The population pharmacokinetic model was built sequentially, starting with a covariate-free model and progressing to a covariate model with the aid of generalized additive modelling. The addition of the covariates weight, body surface area, albumin, serum creatinine, serum urea, alkaline phosphatase and lactate dehydrogenase improved the prediction errors of the model. Typical pretreatment (mean±sem) initial clearance of ifosfamide was 3.03±0.18 L/h with a volume of distribution of 44.0±1.8 L. Autoinduction, dependent on ifosfamide levels, was characterized by an induction half-life of 11.5±1.0 h with 50% maximum induction at 33.0±3.6 µM ifosfamide. Significant pharmacokinetic-pharmacodynamic relationships (p=0.019) were observed between the exposure to 2- and 3-dechloroethylifosfamide and orientational disorder, a neurotoxic side-effect. No pharmacokinetic-pharmacodynamic relationships between exposure to 4-hydroxyifosfamide and haematological toxicities could be observed in this population.

Introduction

Ifosfamide (Holoxan®) is an alkylating agent, used as a single chemotherapeutic drug or in combination schedules. Ifosfamide has been proven to be active against a number of solid tumours and haematological malignancies in adults and children.[1] Large interpatient variability in clinical toxicity and response rates have been observed during ifosfamide treatment.[2] Besides pharmacodynamic variability, this could possibly be explained by

interpatient differences in ifosfamide pharmacokinetics. Ifosfamide is a prodrug, which needs activation by cytochrome P450-3A4 (CYP3A4) to form 4-hydroxyifosfamide, as depicted in figure 1.

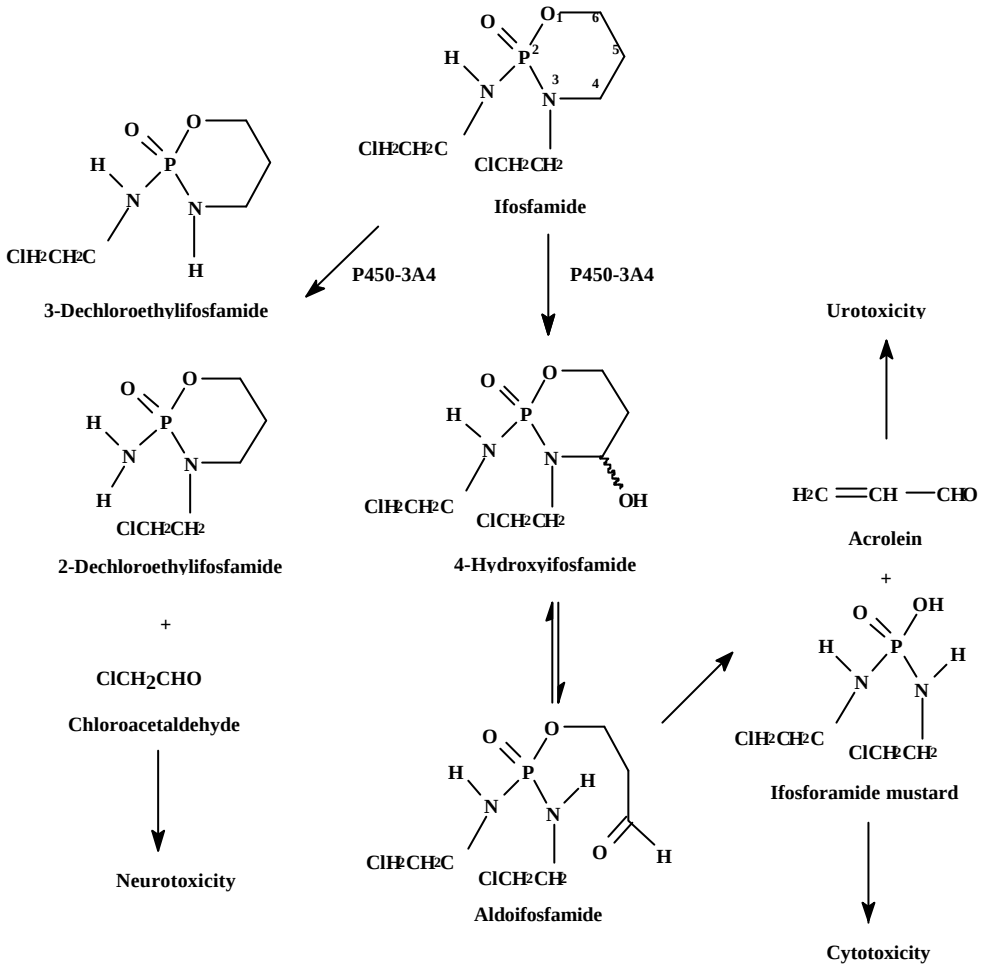

Figure 1. *Metabolism of ifosfamide.*

Intracellular spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard.[1] Therefore, 4-hydroxyifosfamide may be used to describe the pharmacokinetic-pharmacodynamic relationship between the plasma concentrations and the anti-tumour efficacy and toxicity of ifosfamide treatment. Ifosfamide is deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide. Each dechloroethylation reaction yields an equimolar amount of chloroacetaldehyde, which is held responsible for neurotoxicity observed in about 10% of all patients receiving conventional single-agent dosing of ifosfamide.[3] Neurotoxicity can be succesfully treated with the antidote methylene blue. Since chloroacetaldehyde degrades rapidly, assessment of 2- and 3-dechloroethylifosfamide may be preferred when quantifying the relationship between the pharmacokinetics and neurotoxicity after administration of ifosfamide.

Ifosfamide metabolism has been reported to be subject to autoinduction leading to an increase of metabolism of ifosfamide over time.[1] Variability in the rate and the extent of this process may contribute to the large interpatient variability in toxicity and response. As a result

assessment of the population pharmacokinetics of ifosfamide and its metabolites may be important since this may help to interpret the variability.
In this study pharmacokinetics and pharmacodynamics were investigated in patients participating in a phase II study on high dose ifosfamide in advanced soft tissue sarcoma.[4] Aim of our investigation was to develop a population pharmacokinetic model for ifosfamide and its metabolites. The correlations between the patient characteristics and pharmacokinetics were assessed. Furthermore, pharmacokinetic-pharmacodynamic relationships between the 2- and 3-dechloroethylifosfamide and neurotoxicity and between the 4-hydroxyifosfamide and haematological toxicity were investigated.

Methods

Patient selection

Patients were treated for various types of soft tissue sarcoma with single agent high-dose ifosfamide in an open non-randomized multi-centric phase II trial at the Leiden University Medical Center and Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute, Amsterdam, the Netherlands. Eligibility criteria included WHO performance status of 0-2, adequate bone marrow function (white blood cells 4.0 x 10^9/L, platelets 100 x 10^9/L), adequate hepatic function (total bilirubin 30 µmol/L), adequate renal function (serum creatinine 120 µmol/L), no active infection or post-surgical complications, no central nervous system disease and no concomitant second malignant disease. Previous radiotherapy or chemotherapy had to be discontinued for at least 4 weeks. The study protocol was approved by the Ethics Boards of both above mentioned hospitals. Patients were subjected to pharmacokinetic sampling after written informed consent was obtained.

Patient characteristics

Pre-treatment evaluation included a complete medical history and physical examination. These examinations yielded information on patient characteristics such as age (AGE), weight (WT), body surface area (BSA) and gender (SEX). Before treatment, blood chemistry and haematological parameters (haemoglobin, white blood cells and platelets) were evaluated. Blood chemistry yielded baseline levels for sodium, potassium, calcium, urea (UREA), creatinine (CREA), creatinine clearance (CrCL), alkaline phosphatase (AF), lactate dehydrogenase (LDH), alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT), gamma-glutamyltransferase (γ-GT), albumin (ALB) and total protein (TP). CrCL was estimated by the Cockcroft-Gault formula.[5]

Drug administration

Patients received 9 or 12 g/m² ifosfamide (Holoxan®, ASTA Medica, Diemen, The Netherlands) as a 72-hour continuous intravenous infusion once every 4 weeks. Supportive care consisted of mesna and extensive hydration to prevent haemorrhagic cystitis and

bicarbonate to prevent acidosis. Standard 5-HT_3 blockers as anti-emetics and methylene blue (a neurotoxicity antidote) were given when indicated.

Pharmacokinetic sampling

Blood samples were drawn from an indwelling intravenous cannula placed in the arm contralateral to the arm receiving ifosfamide at 0, 3, 10, 20, 24, 34, 48, 58, 68, 72, 73, 76, 80 and 92 hours after start of the infusion. Ten ml of whole blood were collected in a lithium heparin-coated Vacutainer® (Becton-Dickinson, Plymouth, UK) and placed on ice water. The plasma was immediately separated by centrifugation at 1,000 g for 5 min at 4°C. The plasma was aliquoted in three volumes of which two were precisely 1-ml volumes. To these two 1-ml volumes, 100 µl 2 M semicarbazide solution at pH 7.40 were added to stabilize 4-hydroxyifosfamide. The remaining plasma was used for ifosfamide, 2- and 3-dechloroethylifosfamide analysis. Urine was collected for up to 96 hours after the start of the ifosfamide infusion and analysed for ifosfamide, 2- and 3-dechloroethylifosfamide. Both plasma and urine samples were stored at -70°C, pending analysis.

Bioanalysis

Gas chromatography with selective nitrogen-phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[6] Sample pre-treatment consisted of alkalinized liquid-liquid extraction with ethyl acetate, transfer of the organic extract, evaporation to dryness and subsequent reconstitution in ethyl acetate. This method was validated and proved to be specific, sensitive, accurate (93.3-104.5%) and precise (within and between-day <5.5%) within the concentration range of 0.192 to 383 µM, with a lower limit of quantification (LLQ) of 0.192 µM for ifosfamide, 2- and 3-dechloroethylifosfamide.
High-performance liquid chromatography (HPLC) was used for determination of 4-hydroxyifosfamide plasma levels.[7] In brief, this method determined the 4-hydroxyifosfamide-semicarbazone derivative in plasma. Sample pre-treatment consisted of liquid-liquid extraction with ethyl acetate. The HPLC column used was reversed phase C_8 with acetonitrile-0.025 M potassium dihydrogenphosphate (32:68 v/v) as mobile phase. Detection was performed at 230 nm. This method was specific, sensitive, accurate (94.1-107.9%) and precise (within and between-day <7.2%) in the concentration range of 0.361 to 361 µM, with a LLQ of 0.361 µM 4-hydroxyifosfamide.

Data evaluation

Pharmacokinetic models were fitted to the data from the individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling, version V 1.1, double precision, first order estimation).[8,9] The non-linear pharmacokinetics of ifosfamide were described using a recently developed model, which incorporated the development of autoinduction.[10] According to this recent study the pharmacokinetics of ifosfamide can be

described by using a one-compartment model. The change of the amount of ifosfamide (A_{ifo}, µmol) in the central compartment over time can be described by equation 1,

$$\mathbf{t < T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = R - \left(CL \times A_{ifo} / V_{ifo}\right) \text{ and}$$

$$\mathbf{t > T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = -\left(CL \times A_{ifo} / V_{ifo}\right) \qquad \text{(eq. 1)}$$

in which T_{inf} (h) is the infusion duration, R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL (L· h^{-1}) the ifosfamide clearance, which changes over time and V_{ifo} (L) the volume of distribution. The concentration of ifosfamide C_{ifo} (µM) is the ratio of A_{ifo} and V_{ifo}. In turn, autoinduction can be described using a hypothetical enzyme compartment. CL can then be expressed by an initial clearance ($CL_{ini,ifo}$, L· h^{-1}) multiplied by the relative amount of enzyme (A_{enz}) in the hypothetical enzyme compartment, as given in equation 2.

$$CL = CL_{ini,ifo} \times A_{enz} \qquad \text{(eq. 2)}$$

The change of A_{enz} (no dimension) over time in the enzyme compartment is dependent on C_{ifo} as follows,

$$\frac{dA_{enz}}{dt} = K_{enz,out} - K_{enz,out} \times A_{enz} \times \left(1 - \frac{C_{ifo}}{C_{ifo} + IC_{50}}\right) \qquad \text{(eq. 3)}$$

in which $K_{enz,out}$ (h^{-1}) is the first-order rate constant for enzyme degradation/inactivation and IC_{50} (µM) is the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation. The induction half-life of the enzyme ($T_{½enz}$, h) was calculated by the ratio of ln(2) and $K_{enz,out}$.

The pharmacokinetics of ifosfamide and metabolites were described sequentially. Bayesian estimations (posthocs) of the pharmacokinetic parameters of ifosfamide were used for the description of the pharmacokinetics of the metabolites. The change in the amount of a metabolite (A_m) over time could be described by equation 4.

$$\frac{dA_m}{dt} = \left(F_m \times CL \times \frac{A_{ifo}}{V_{ifo}}\right) - \left(K_m \times A_m\right) \qquad \text{(eq. 4)}$$

In which K_m (h^{-1}) is the elimination rate constant of the metabolite and F_m is the fraction of ifosfamide metabolized to the metabolite. The values for F_m and the volume of distribution of the metabolite (V_m, L) cannot be estimated separately in this model. Therefore, the ratio of F_m over V_m was estimated: F^* (L^{-1}).

The residual or intra-individual variability of ifosfamide and metabolites kinetics were described separately with a proportional and additive term. The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model.

The posthocs of the areas under the concentration-time curves (AUC) of ifosfamide and its metabolites were obtained by describing the cumulative concentrations of these compounds over a given time. AUCs were obtained with extrapolation of ifosfamide and metabolite concentration-time profiles to 144 hours after the start of the infusion, where concentrations were below the LLQ. The urinary excretions (UE) for ifosfamide and 2- and 3-dechloroethylifosfamide were calculated as the equimolar amount of the administered ifosfamide dose recovered in urine over 96 hours.

Covariate model building

The patient characteristics and blood chemistry parameters prior to the ifosfamide infusion were analysed for their correlations with the pharmacokinetic parameters of ifosfamide and its metabolites. The influence of these covariates was tested using the Generalized Additive Modelling (GAM) procedure in the software programme Xpose (Xpose, version 2.0, Uppsala University, Sweden) as implemented in the statistical programme S-PLUS (version 2000 Professional release I, Mathsoft).[11] Covariates that correlated significantly with the pharmacokinetic parameters, as indicated by the Akaike criterion, were selected for testing in NONMEM.[12] The selected covariates were entered stepwise into the structural NONMEM model.[13] Continuous variables were centred to their medians, as depicted in equation 5,

$$P_{pop} = \theta_1 + \theta_2 \times (COV_{ind} - COV_{med}) \quad \text{(eq. 5)}$$

in which, P_{pop} is a population pharmacokinetic parameter, θ_1 the typical value of P_{pop}, θ_2 the influence of the covariate on P_{pop}, COV_{ind} the individual value of the covariate and COV_{med} the median value of that covariate. An increase in goodness-of-fit caused by the introduction of a covariate, is associated with a decrease in the value of the objective function. The objective function of the NONMEM model is approximately chi-squared distributed and a decrease of > 3.8 upon entering a covariate was considered statistically significant ($p<0.05$).[9] All variables that caused significant decreases in objective function were entered into the intermediate model. A stepwise backward elimination procedure was then performed with all variables entered in the intermediate model. Variables were retained in the final model when elimination of the variable caused an increase in objective function > 7.8 ($p<0.005$). Individual pharmacokinetic parameters for ifosfamide were calculated with the final model using the posthoc option in NONMEM. Subsequently, identical procedures were followed for 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide population models.

Pharmacodynamics

Haematological assessment yielded nadirs for haemoglobin (HGB), white blood cells (WBC) and platelets (PLT). The haematological toxicities were evaluated using the percentage decrease in HGB, WBC and PLT. The percentage decrease was calculated using equation 6,

$$\%\ \text{decrease} = \frac{\text{Pretreatment value} - \text{value of nadir}}{\text{Pretreatment value}} \times 100\% \quad \text{(eq. 6)}$$

Non-haematological toxicity such as, nausea, vomiting, anorexia, alopecia, fatigue, constipation, diarrhoea, fever, pain, irritation, activity score, peripheral neurotoxicity, central neurotoxicity, such as loss of consciousness, concentration problems, orientation disorder, hallucinations and coma were scored with a dichotomous scale (absent or present during the study period), at the discretion of the physician.

Pharmacokinetic-pharmacodynamic relationships

Correlations between the pharmacokinetics and pharmacodynamics were explored using scatter plots and Pearson´s correlation coefficients (r). Correlations between the pharmacokinetic parameters and categorical toxicity data were explored using the Spearman rank correlation test and logistic regression. Statistical analysis was performed using SPSS

software (Version 6.1 for Windows; SPSS, Inc., Chicago, IL, USA). The level of significance was set at $p<0.05$. All tests for significance were two-tailed.

Results

Patients and treatment

A total of 20 patients was enrolled in the pharmacokinetic study. Six patients received 12 g/m² ifosfamide as a 72-hour continuous intravenous infusion once every 4 weeks. Due to unacceptable central neurotoxicity in this group, the following 14 patients received an adjusted dose of 9 g/m²/72 h. The infusion of 12 g/m² in one patient was stopped after 48 hours due to acute severe neurotoxicity during drug-administration. Therefore, this patient received only 66% of the planned dose. Five patients received methylene blue after neurotoxicity had occurred. In addition, two patients received prophylactic methylene blue, because neurotoxicity occurred in the previous treatment course. Additional co-medication included anti-coagulants, H_2 receptor antagonists, glucocorticosteroids and tricyclic anti-depressants. The patient characteristics and blood chemistry are summarized in table 1.

Table 1. *Characteristics of 20 patients receiving 9 or 12 g/m² ifosfamide by means of a 72-hour continuous i.v. infusion and blood chemistry values prior to study entry.*

Characteristics	mean (range)	Blood chemistry	median (range)	reference values
No. of patients	20	Sodium (mM)	140 (138-146)	135-148
Sex (M/F)	14/6	Potassium (mM)	4.4 (3.8-5.1)	3.5-5
Median age (years)	49 (24-72)	Calcium (mM)	2.3 (2.2-2.4)	2.2-2.7
Median weight (Kg)	59 (49-88)	Urea (mM)	5.1 (2.6-8.7)	2.9-8.9
Body surface area (m^2)	1.75 (1.43-2.09)	Creatinine (µM)	77 (53-101)	50-133
Prior Radiotherapy	4	Creatinine clearance (ml/min)	82 (51-144)	> 60
Prior Surgery	20	Albumin (g/L)	43 (36-77)	30-52
Prior chemotherapy	14	Total protein (g/L)	73 (34-81)	60-84
Metastases	17	AF (U/L)	147 (68-250)	100-280
Primary tumour		LDH (U/L)	377 (166-1123)	150-300
Rhabdomyosarcoma	3	ALAT (U/L)	26 (7-72)	10-32
Neurofibrosarcoma	1	ASAT (U/L)	28 (14-61)	10-32
Osteosarcoma	3	γ-GT (U/L)	61 (15-143)	< 40 (M) <25 (F)
Synoviumsarcoma	3			
Leiomyosarcoma	6			
Endometrial sarcoma	2			
Liposarcoma	2			

Pharmacokinetics

Pharmacokinetic assessment resulted in 228, 230, 228 and 119 concentration-time points for ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide, respectively. Pharmacokinetic profiles showed initial build-up of ifosfamide plasma concentrations in the first 24 hours (reaching maximum concentrations), followed by a gradual decrease due to autoinduction. After 72 hours the infusion was stopped and elimination pharmacokinetics were observed. A one-compartment model with a hypothetical enzyme compartment adequately characterized the time profiles of the ifosfamide plasma concentrations. The pharmacokinetic parameters of ifosfamide were related to various combinations of covariates. GAM-analysis indicated significant correlations between UREA (µM), CREA (µM) and $CL_{ini,ifo}$, WT (Kg) and V_{ifo}, AF ($U \cdot L^{-1}$) and LDH ($U \cdot L^{-1}$) and $K_{enz,out}$, and CREA (µM) and IC_{50}. After forward inclusion and backward elimination of these covariates, a final population model for ifosfamide was obtained. Significant correlations were obtained for the covariates included in equations 7-9.

$$V_{ifo} = 44.0 + 0.56 \times (WT - 59) \quad \text{(eq. 7)}$$

$$CL_{ini,ifo} = 3.03 + 0.169 \times (UREA - 5.1) - 0.0267 \times (CREA - 77.2) \quad \text{(eq. 8)}$$

$$K_{enz,out} = 0.0602 + 0.0000647 \times (AF - 147) \quad \text{(eq. 9)}$$

Conform equation 5, each population parameter was described by an estimate of the typical value of that parameter plus an estimate of the influence of the covariate. The covariate was centred to its median value, eg. for V_{ifo}, a typical value for a patient of 59 kg is 44 L. Increase of WT with 1 kg results in an increase of V_{ifo} with 0.56 L. WT ranged between 49 and 88 kg,

hence V will range between 38 and 60 L. Inclusion of the covariates resulted in a decrease in objective function of 41 in comparison with the intermediate model without covariates. Bayesian estimates of the pharmacokinetic parameters of ifosfamide were obtained. These parameters were used for the development of pharmacokinetic models for the metabolites. A sequential approach was used, because the data did not allow simultaneous estimation of the population pharmacokinetic parameters of ifosfamide and its metabolites.

No interindividual variability could be estimated for the elimination rates of the metabolites. Hence, no correlations with covariates could be found for these parameters. This should not be interpreted as an absence of interindividual variability, but only that the data do not contain sufficient information to estimate the interindividual variability of the metabolite elimination rates.

Table 2. *Covariate-based estimates of population pharmacokinetic parameters for ifosfamide (IFO n=20), 2-dechloroethylifosfamide (2DCE n=20), 3-dechloroethylifosfamide (3DCE n=20) and 4-hydroxyifosfamide (4OHIF n=11) with their relative standard error of the mean, interindividual variability and residual variability, obtained in patients receiving 9 or 12 g/m² ifosfamide by means of a 72-hour continuous i.v. infusion.*

Parameter	Mean	Rel. err.	I.I.V.	R.V.	Mean	Rel. err.
$CL_{ini,ifo}$ (L· h^{-1})	3.03	6%	13%	P.E. IFO	13.7%	11%
θ_{UREA}	0.169	32%		A.E. IFO (µM)	0.0606	43%
θ_{CREA}	0.0267	44%				
V_{IFO} (L)	44.0	4%	13%			
θ_{WT}	0.56	25%				
$K_{enz,out}$ (h^{-1})	0.0602	9%	37%			
θ_{AF}	0.0000647	19%				
$T_{½enz}$ (h)	11.5					
IC_{50} (µM)	30.0	12%	10%			
F^*_{2DCE} (L^{-1})	0.0140	32%	22%	P.E. 2DCE	-	
θ_{ALB}	0.000413	40%		A.E. 2DCE (µM)	1.95	4%
θ_{BSA}	0.0164	100%				
K_{2DCE} (h^{-1})	0.501	30%	-			
F^*_{3DCE} (L^{-1})	0.0745	130%	22%	P.E. 3DCE	35.6%	37%
K_{3DCE} (h^{-1})	1.51	130%	-	A.E. 3DCE (µM)	2.51	13%
F^*_{4OHIF} (L^{-1})	0.338	21%	55%	P.E. 4OHIF	33.0%	30%
θ_{LDH}	0.000773	48%		A.E. 4OHIF (µM)	0.228	43%
K_{4OHIF} (h^{-1})	168	26%	-			

Rel. Err.=Relative error of the mean, I.I.V.=interindividual variability, R.V.=residual variability, $CL_{ini,ifo}$=initial ifosfamide clearance, θ=covariate, UREA=urea serum concentration, CREA=creatinine serum concentration, V_{IFO}=volume of distribution of ifosfamide, WT=weight, $K_{enz,out}$=autoinduction formation rate, AF=alkaline phosphatase, $T_{½,enz}$=induction half-life of the enzyme, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, P.E.=proportional intraindividual error, A.E.=additive intraindividual error, F^*=ratio of fraction metabolized and volume of distribution of metabolite, ALB=albumin, BSA=body surface area, K=first-order rate constant for metabolite elimination, LDH=lactate dehydrogenase.

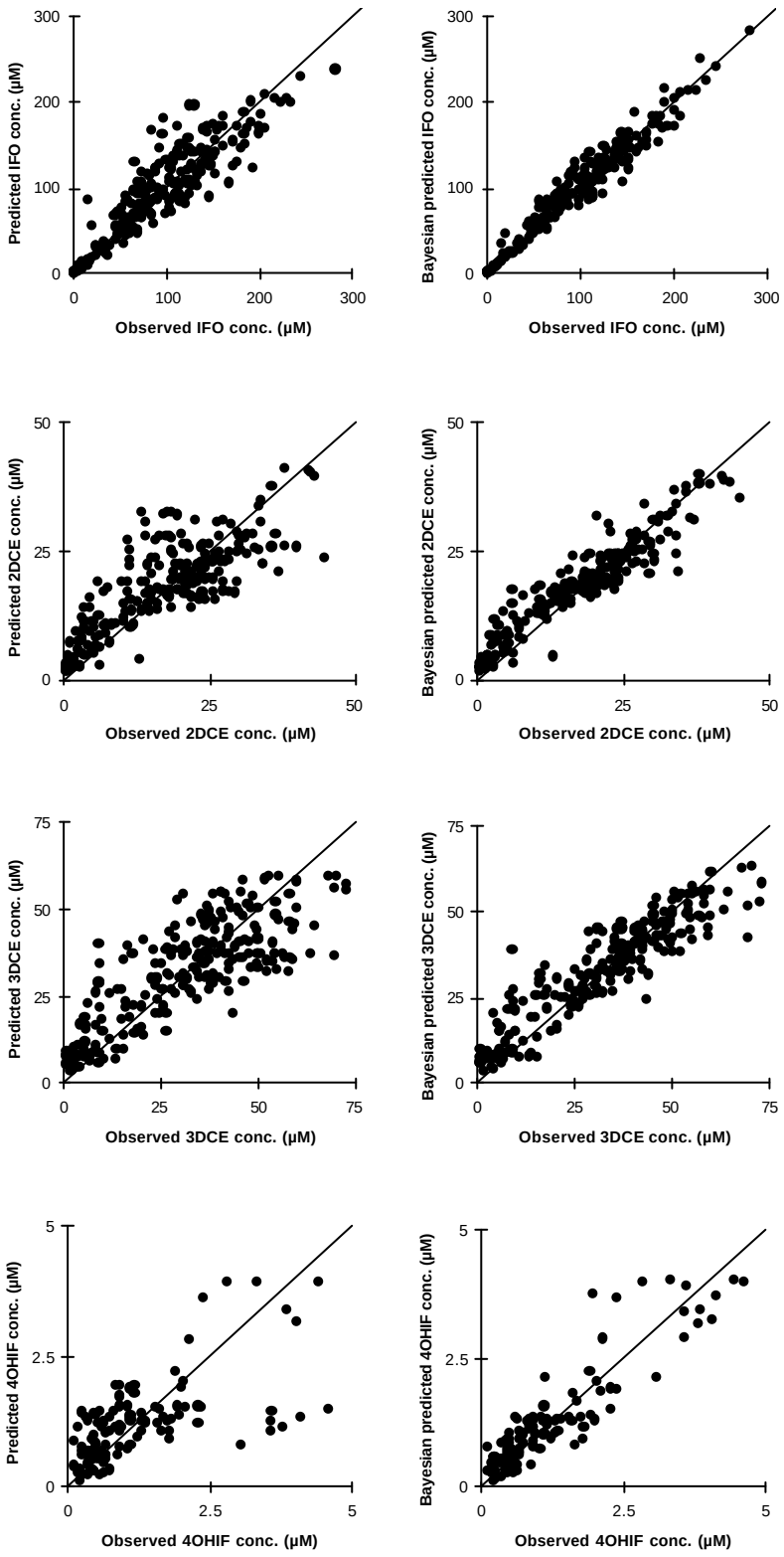

Figure 2. *The relationships between the final model predictions, individual Bayesian estimates and observed ifosfamide, 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) concentrations.*

GAM-analysis of the Bayesian estimates of the metabolites indicated BSA (m^{-2}) and ALB ($g \cdot L^{-1}$) for F^*_{2DCE}, AGE (y), LDH and UREA (µM) for F^*_{3DCE} and LDH ($U \cdot L^{-1}$) for F^*_{4OHIF} to be significant covariates. After forward inclusion and backward elimination of these covariates, a final population model for the metabolites was obtained. Significant correlations were obtained for the covariates included in equations 10-11.

$$F^*_{2DCE} = 0.0140 + 0.000413 \times (ALB - 43) - 0.0164 \times (BSA - 1749) \quad \text{(eq. 10)}$$

$$F^*_{4OHIF} = 0.338 + 0.000773 \times (LDH - 377) \quad \text{(eq. 11)}$$

Inclusion of these covariates resulted in a decrease in objective function of 16 and 18 for 2-dechloroethylifosfamide and 4-hydroxyifosfamide models compared to their respective intermediate models without covariates. Estimates of pharmacokinetic population parameters of the final model with their standard error and their interindividual variability and the residual variability are presented in table 2. The relationships between the observed and population predicted concentrations and between the observed and Bayesian individually predicted concentrations of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide, as described by the final covariate model, are depicted in figure 2.

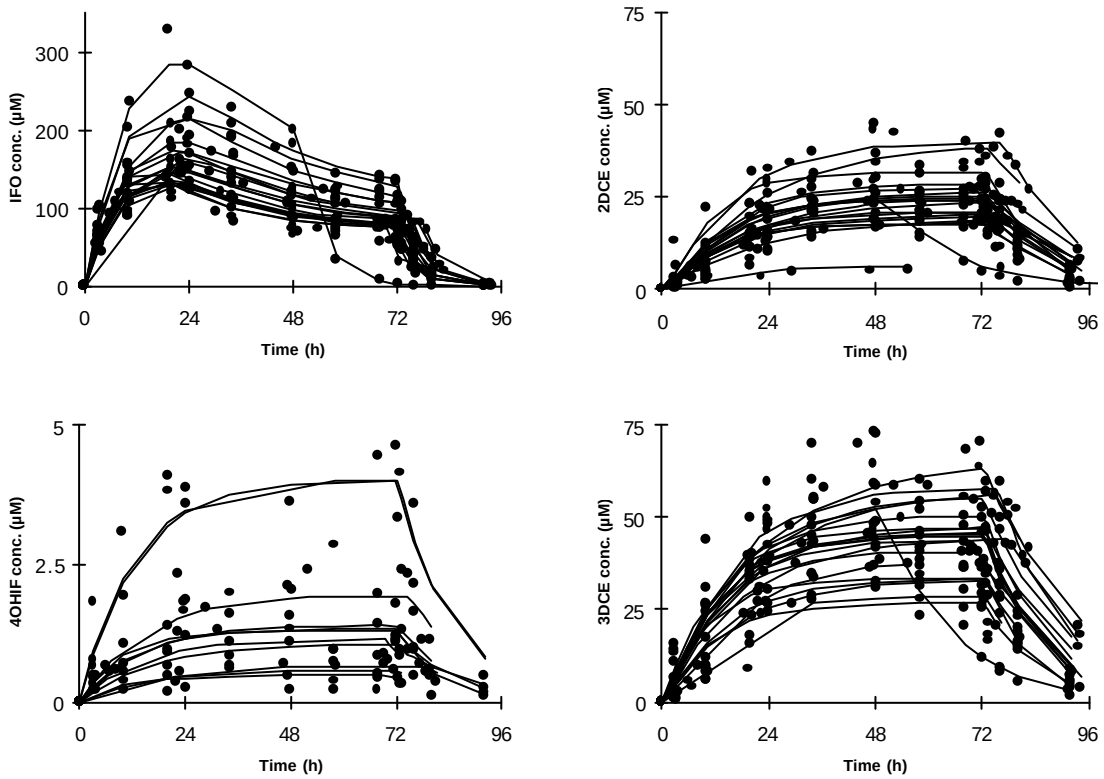

Figure 3. *The concentration time profiles of ifosfamide (IFO), 2dechloroethylifosfamide (2DCE), 3 dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF). The observations are represented by the dots and the Bayesian individual predictions are connected by the lines.*

The observed and Bayesian estimated pharmacokinetic profiles for ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide of all patients are represented in figure 3. No outliers or trends could be observed in the plots of the weighted residuals versus the predicted concentrations and the individual weighted residuals versus time (not shown).
The time profile of clearance (CL) is described in figure 4. Initial clearance ($CL_{ini,ifo}$ = 3.03 L/h) increased three-fold during the infusion and declined to pre-treatment levels after cessation of the infusion. The pharmacokinetic model described the increase in clearance as a progressively developing effect with a half-life of 11.5 h. The interindividual variability in clearance was moderate. At the start of the infusion the posthoc estimations for $CL_{ini,ifo}$ ranged from 2.3 to 4.3 L/h and at the end of the infusion from 6.5 to 14.7 L/h.

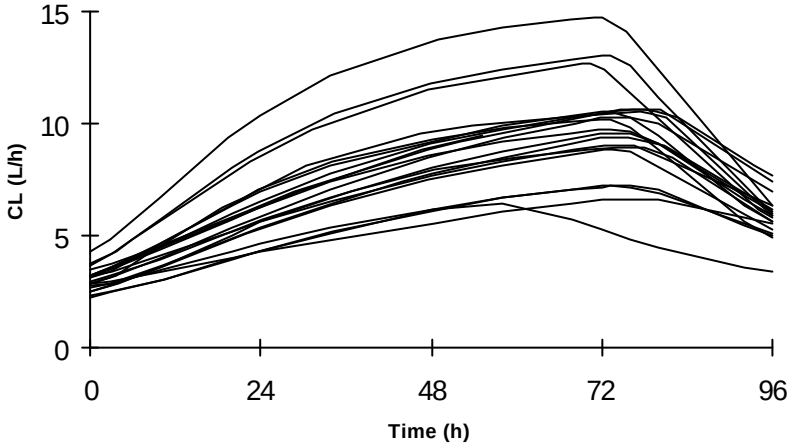

Figure 4. *Individual time profiles of the ifosfamide clearance (CL) for all patients (n=20). The profiles were obtained on basis of the individual Bayesian parameter estimates provided by NONMEM.*

Mean (and range) of the Bayesian estimates of the AUC of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were 9.30 (7.11-13.62), 1.72 (0.49-3.01), 3.18 (1.92-4.64) and 0.117 (0.036-0.290) mM· h, respectively. The plots of AUCs of ifosfamide and its metabolites versus ifosfamide dose are presented in figure 5. The plots indicate that the exposure of ifosfamide and its metabolites increases with increasing dose. Table 3 contains the urinary excretion data of ifosfamide and 2- and 3-dechloroethylifosfamide for all individuals.

Table 3. *Mean urinary excretion (UE) and coefficient of variation (CV) of ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide (3DCE).*

	UE	CV
IFO	19.9%	35%
2DCE	9.3%	28%
3DCE	17.0%	47%

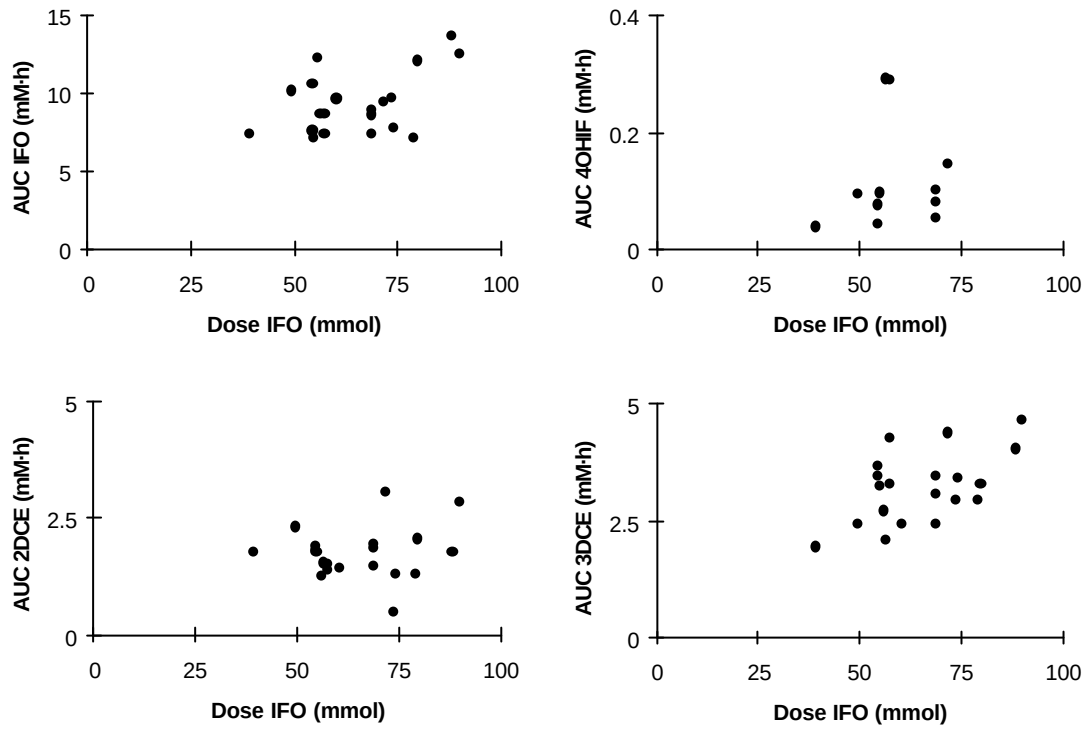

Figure 5. *The relationship between the absolute ifosfamide dose and ifosfamide, 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) exposures as calculated by the area under the concentration-time curve (AUC) of each patient.*

Pharmacodynamics

The median percentages decrease in HGB, WBC and PLT and frequencies of the non-haematological toxicities (e.g. neurotoxicities and gastrointestinal toxicities) are represented in table 4.

Table 4. *Median percentage change in haemoglobin (HGB), white blood cells (WBC) and platelets (PLT) and frequencies (absent or present during treatment) of non-haematological toxicities in 20 patients receiving 9 or 12 g/m² ifosfamide by means of a 72-hour continuous intravenous infusion.*

Haematological toxicity	Median % change (range)	Frequency of gastrointestinal toxicity	
HGB	-17% (-48% - +15%)	Nausea	12/20
WBC	-76% (-99% - +9%)	Vomiting	10/20
PLT	-13% (-82% - +64%)	Anorexia	10/20
Frequency of neurotoxicity		Constipation	11/20
Central neurotoxicity	6/20	Diarrhoea	2/20
Vision distortion	4/20	Frequency of other toxicities	
Loss of consciousness	1/20	Fever	1/20
Concentration problems	3/20	Pain	5/20
Hallucinations	2/20	Alopecia	9/20
Orientational disorder	2/20		

Pharmacokinetic-pharmacodynamic relationships

All statistical significant correlations between the pharmacokinetic and pharmacodynamic parameters with their level of significance and correlation coefficient are represented in table 5.

Table 5. *Statistical significant (p<0.05) linear correlations between pharmacokinetic and pharmacodynamic parameters, obtained in 18 patients receiving 9 or 12 g/m² ifosfamide by means of a 72-hour continuous i.v. infusion.*

Pharmacokinetics	Effect	Statistical method	p-value	R^2
IC_{50} (µM)[#]	HGB (% decrease)	Pearson´s	0.009	-0.58
AUC_{2DCE} (h· mM)	Orientation disorder	Spearman-rank	0.019	0.53
AUC_{3DCE} (h· mM)	Orientation disorder	Spearman-rank	0.019	0.53
F^*_{3DCE}	Orientation disorder	Spearman-rank	0.038	0.56
AUC_{2DCE} (h· mM)	Vision distortion	Multiple logistic regression	0.013	
and	Concentration problems	Multiple logistic regression	0.006	
AUC_{3DCE} (h· mM)	Loss of consciousness	Multiple logistic regression	0.004	
	Orientation disorder	Multiple logistic regression	0.003	
	Hallucinations	Multiple logistic regression	0.003	

[#]=in 20 patients, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, HGB=haemoglobin, 2DCE=2-dechloroethylifosfamide, 3DCE=3-dechloroethylifosfamide, AUC=area under the plasma concentration-time curve, F^*=ratio of fraction metabolized and volume of distribution of metabolite.

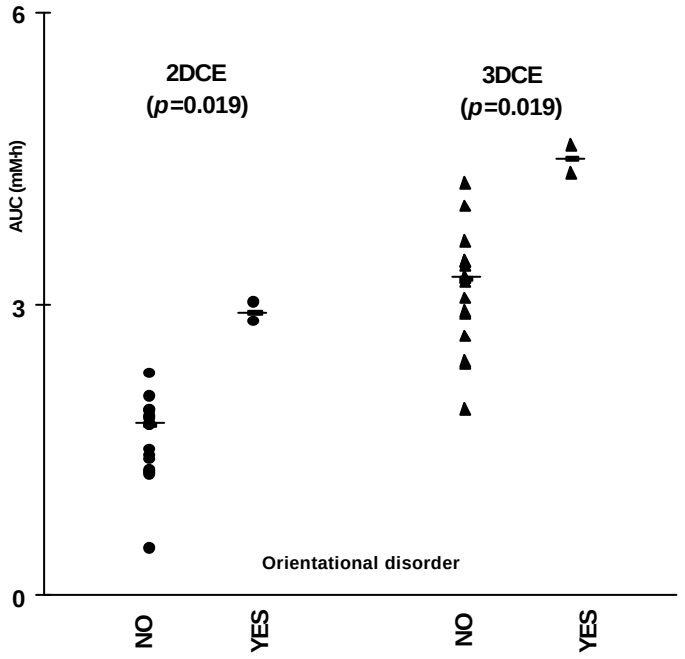

Figure 6. *The relationship between the neurotoxic side-effect orientational disorder and the area under the concentration-time curve (AUC) of 2-dechloroethylifosfamide (2DCE) and 3-dechloroethylifosfamide*

(3DCE). Level of significance calculated by Spearman-rank test.

The two patients with prophylactic methylene blue treatment for neurotoxicity were excluded from the pharmacodynamic evaluation. Multiple logistic regression indicated correlations between the exposure to 2- and 3-dechloroethylifosfamide with central neurotoxic side-effects including vision distortion, concentration problems, loss of consciousness, orientation disorder and hallucinations. Except for orientational disorder, these correlations were not observed with either the exposure to 2- or 3-dechloroethylifosfamide alone. The pharmacokinetic-pharmacodynamics relationship between the exposure to the dechloroethylated metabolites and orientational disorder, a central neurotoxic side-effect, is represented in figure 6. No relationship between the exposure to ifosfamide or 4-hydroxyifosfamide and the haematological toxicities was observed.

Discussion

In this study the population pharmacokinetics of ifosfamide and its metabolites and the pharmacokinetic-pharmacodynamic relationships were assessed in patients who participated in a multicentre phase II study on high dose ifosfamide in advanced soft tissue sarcomas.[4] In this study a partial response rate of only 16% was seen with severe toxicity resulting in a negative recommendation for this indication. Nevertheless, knowledge of the population pharmacokinetics and pharmacokinetic-pharmacodynamic relationships may be beneficial for other clinical applications of ifosfamide. The population pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were adequately described by the model (table 2, figure 4). Modest interindividual differences in the parameters were observed. Interindividual variability in the pharmacokinetic parameters of ifosfamide ranged between 10% and 37%. The metabolite pharmacokinetics demonstrated larger differences with interindividual variability ranging between 22% and 55%, which has been observed before in population pharmacokinetic models which included metabolites.[14] Residual variability was also acceptable for ifosfamide and its metabolites.

The inclusion of the selected covariates resulted in an improved goodness-of-fit of the population pharmacokinetic model. The relationship between weight and V_{ifo} is self-explicatory. An analogous relationship with F^*_{2DCE} and BSA was found. F^*_{2DCE} is the ratio of the fraction metabolized and the volume of distribution of 2-dechloroethylifosfamide. BSA depends on both the characteristics height and weight. Thus, with increasing weight V_{2DCE} will increase and F^*_{2DCE} will decrease. CREA and UREA are both indicators of renal function: increased levels indicate reduced function. In the final model the relationship between renal function and $CL_{ini,ifo}$ is not clear since correlations between $CL_{ini,ifo}$ and CREA and UREA had opposite signs. Moreover, no relationship with CrCL was found. No plausible physiological explanation can be given for the other significant correlations between patient characteristics and pharmacokinetics of ifosfamide and metabolites.

Renal excretion of unchanged ifosfamide amounted to 20%. Furthermore, a model with separate estimation of renal and metabolic clearance did not produce a decrease in objective function. This indicates that renal clearance does not constitute a major route of elimination of ifosfamide. Previously, similar urinary excretions were found. Gillard et al. observed renal

excretion of ifosfamide, 2- and 3-dechloroethylifosfamide of 18, 16 and 7%, respectively, in patients receiving 3g/m²/3 hours ifosfamide for 3 days.[15]

The volumes of distribution of the metabolites (V_m) may be estimated on basis of their respective F^* values. F^* represents the ratio of F_m (fraction of ifosfamide metabolized to the metabolite) and V_m. On basis of the data presented in table 3 F_m of 3-dechloro-ethylifosfamide ranges from 0.17 to 0.708 corresponding to a V_m range of 2.3 to 9.5 L. The lower boundary of F_m is based on the urinary excretion of 3-dechloroethyl-ifosfamide. The upper boundary of F_m is based on fraction of the ifosfamide dose not excreted in the urine as 3-dechloroethylifosfamide. Corresponding V_m values of 2-dechloroethylifosfamide range from 6.6 to 45 L. These calculated volumes of distribution are consistent with the expectation that in general V_m´s will be less than V of the parent compound, because detoxification tends to lead to more hydrophilic compounds.

In our study, one patient developed acute severe neurotoxicity during drug administration. Interestingly, the ifosfamide concentrations in this patient were the highest in the population, but the dechloroethylated metabolite profiles were not different from the rest of the population. The relatively low clearance of ifosfamide in this patient should theoretically result in a lower AUC of neurotoxic metabolites and less neurotoxicity. The latter is in contrast with the clinical observations. It can therefore be concluded that the development of neurotoxicity cannot be predicted or explained on the basis of the pharmacokinetic parameters of ifosfamide alone.

In a previous study it was demonstrated that autoinduction of metabolism causes non-linear pharmacokinetics of ifosfamide: increase in ifosfamide dose results in a less than proportional increase in AUC of ifosfamide.[10] Consequently, the AUCs of the metabolites would increase more than proportional with an increase in ifosfamide dose. In the current study, however, these trends cannot be observed in the plots, possibly because of the interindividual variability (figure 5).

In contrast with our findings Cerny et al. have reported a linear increase of AUC for ifosfamide from 9.7 to 16.6 mM· h when dose increased from 12 to 18 g/m².[16] Furthermore, AUC of the metabolites did not increase with dose. There is no apparent explanation for the differences in observations by Cerny and co-workers and our findings. However, in our study different structural models (linear and nonlinear) for the description of the concentration-time profiles of ifosfamide and its metabolites were tested. Inclusion of autoinduction in the pharmacokinetic model resulted in an impoved description of the data than a linear pharmacokinetic model.

Several significant pharmacokinetic-pharmacodynamic relationships were observed (table 5). The significant relationship between IC_{50} (the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation) and the percentage decrease from pre-treatment levels of HGB may be explained physiologically. Patients with high IC_{50} values exhibit less autoinduction. Relatively less autoinduction of ifosfamide clearance will result in lower AUCs of 4-hydroxyifosfamide. In theory, lower AUCs of 4-hydroxyifosfamide should correlate with less decrease in HGB levels. However, no significant relationships between the exposure to 4-hydroxyifosfamide and any haematological toxicity was observed, nor has this ever been reported before, thus far.[1] This is probably due to high interindividual variability in pharmacodynamic effect and an insufficient number of patients. Moreover, nadirs are inherently more variable, because of infrequent sampling of haematological parameters.

The exposures to 2- and 3-dechloroethylifosfamide were both positively correlated with the development of orientational problems during the infusion. The F^* of 3-dechloroethyl-ifosfamide was also positively correlated indicating that formation of dechloroethylated metabolites, which is accompanied by the formation of the neurotoxic chloroacetaldehyde is responsible for the observed neurotoxicity. Orientational disorder is characterized by transient lack of recognition of one's temporal and spatial relationships and environment. This form of neurotoxicity can be very disturbing for patients and can have a severe impact on their experience of quality of life. The effect of prophylactic methylene blue administration on the assessment of the pharmacokinetic-pharmacodynamic relationship was taken into account, by excluding patients with prophylactic methylene blue treatment from this analysis. All patients who developed neurotoxicity during the ifosfamide infusion received methylene blue on the second or third day of treatment.

In conclusion, a pharmacokinetic model for ifosfamide and metabolites was developed. The addition of covariates to the population model improved the prediction errors. Moderate interindividual variability in the pharmacokinetics and considerable interindividual variability in the pharmacodynamics was observed. Exposure to both dechloroethylated metabolites was positively and significantly correlated with orientation disorder during ifosfamide therapy. This may be explained by the simultaneous formation of chloroacetaldehyde. No pharmacokinetic-pharmacodynamic relationship between exposure to 4-hydroxyifosfamide and haematological toxicities could be observed in this population.

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Chapter 4.2

Sparse data population pharmacokinetics of ifosfamide and its dechloroethylated and hydroxylated metabolites in children

Summary

The purpose of this study was to assess the feasibility of a sparse data approach for the determination of the population pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4hydroxyifosfamide in children. The sparse data population pharmacokinetic assessment was performed in children treated with single agent ifosfamide against various malignant tumours. The analysis included 32 patients aged between 1 and 18 years receiving a total of 45 courses of 1.2, 2 or 3 g/m² ifosfamide in 1 or 3 hours on 1, 2 or 3 days. Plasma concentrations of ifosfamide and its dechloroethylated metabolites were determined using gas chromatography. Plasma concentrations of 4-hydroxyifosfamide were described using high-performance liquid chromatography. The models were fitted to the data using a non-linear mixed effects model as implemented in the NONMEM programme. Cross-validation was performed by bootstrapping the data. Pharmacokinetic assessment resulted in 133 ifosfamide, 106 2-dechloroethylifosfamide, 116 3-dechloroethylifosfamide and 76 4 hydroxyifosfamide observed concentrations. The model predicted all population pharmacokinetic parameter means, their accuracy, interindividual variability and residual variability. Cross-validation demonstrated no bias. In conclusion, a model to estimate ifosfamide and metabolite concentrations in a sparse data paediatric population was developed and validated.

Introduction

Ifosfamide (Holoxan®) is a cytostatic agent that exerts its effect through DNA alkylation. Phase II clinical trials in paediatric populations have demonstrated activity in a wide range of

tumour types. Ifosfamide is presently included in combination chemotherapy for several tumours, including Ewings sarcoma, osteosarcoma, rhabdomyosarcoma, and other soft tissue sarcomas.[1] Adverse effects of ifosfamide include myelosuppression, nausea and vomiting, alopecia and urotoxicity. The latter can be prevented by the co-administration of mesna. Although encephalopathy is more frequently observed in adults than in children, the consequences of encephalopathy can be more disastrous in children.[2] Ifosfamide can cause chronic nephrotoxicity in children, which can lead to rickets and growth retardation due to renal tubular acidosis. Both the efficacy of ifosfamide treatment and its side effects are highly unpredictable. Some patients experience treatment restricting toxicities, while others do not. Similarly, some tumour types are chemosensitive and curable, whereas others remain resistant or recur following an initial response.[3]

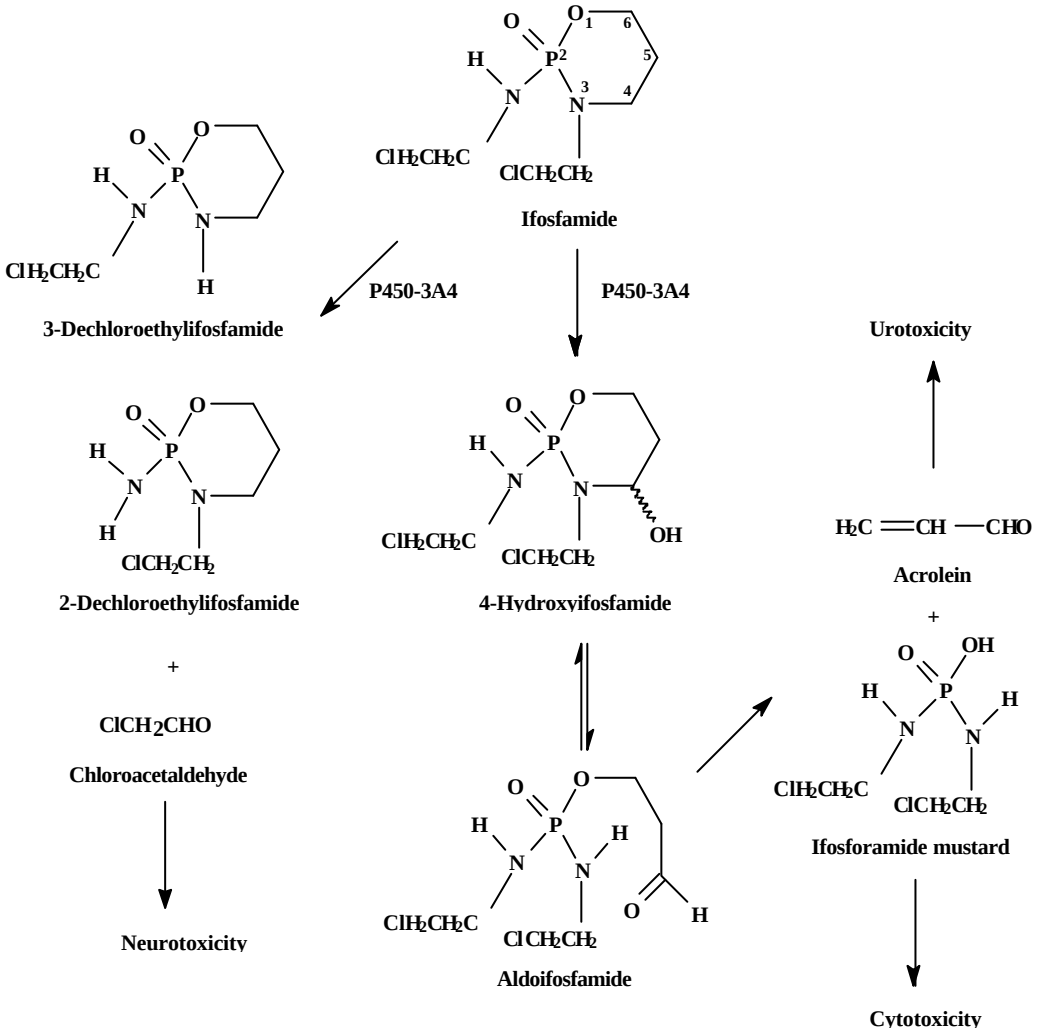

Figure 1. *Metabolism of ifosfamide.*

Ifosfamide is a prodrug, which needs activation by cytochrome P450-3A4 (CYP3A4) to 4-hydroxyifosfamide, as depicted in figure 1. Intracellular spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard.[1] This means that the 4-hydroxyifosfamide is the preferred metabolite to describe the pharmacokinetic-pharmacodynamic relation between the conveniently obtainable plasma concentrations and the anti-tumour efficacy and toxicity of ifosfamide treatment. Ifosfamide is

deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide. Dechloroethylation yields an equimolar amount of the chloroacetaldehyde, which is held responsible for neurotoxicity observed in approximately 10% of all patients receiving conventional single-agent dosing of ifosfamide.[3] Chloroacetaldehyde is rapidly degraded in plasma with a half-life of 2-3 minutes. Therefore, measurement of 2- and 3-dechloroethylifosfamide is preferred in describing the relationship between the pharmacokinetics and the risk of neurotoxicity of ifosfamide treatment. The metabolism of ifosfamide is subject to extensive autoinduction.[1]

Since the efficacy and specific toxicities of the treatment with the prodrug ifosfamide can be linked to its extensive metabolism, pharmacokinetic assessment of these metabolites are of special interest in helping to explain the unpredictable chances of success and failure in ifosfamide treatment. Nowadays, the establishment of these pharmacokinetic-pharmacodynamic relationships are fully standardized in new drug development. Nevertheless, less than a dozen pharmacokinetic trials in children have been conducted to asses the pharmacokinetics of ifosfamide and its metabolites. Boddy et al. especially contributed much of the current knowledge of paediatric ifosfamide pharmacokinetics and its variability.[3-6] A minimal impact on the quality of life of the children can be obtained by applying a sparse data approach limiting the pharmacokinetic assessment to routine phlebotomies and indwelling catheters.[7] The aim of the current study was to assess the feasibility of a sparse data approach for the determination of the population pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide in a paediatric population.

Methods

Patients

A group of 32 paediatric patients suffering from various malignant diseases were subjected to pharmacokinetic sampling during ifosfamide treatment. Inclusion and exclusion criteria were identical to those of the regular ifosfamide treatment. The study protocol was approved by the Ethics Board of the Academic Medical Center of Amsterdam. Written informed consent was obtained from the carer or patient (if more appropriate) before entering the study protocol.

Pharmacokinetic sampling

The pharmacokinetic analysis included 32 patients with 16 males and 16 females aged between 1 and 18 years receiving a total of 45 courses of 1.2, 2 or 3 g/m² ifosfamide in 1 or 3 hours on 1, 2 or 3 days. The Ethics Board approved the study with the understanding that no additional phlebotomies beyond those considered part of routine patient care would be performed. In most patients an already indwelling double-lumen catheter was used. Whole blood was drawn and immediately placed on ice water. A volume of 3 ml whole blood was immediately transferred to a polypropylene tube containing 300 µl 2 M semicarbazide to stabilize 4-hydroxyifosfamide. The whole blood was separated by centrifugation at 1,000 g for 5 min at 4°C. Plasma was transferred and stored at -20°C up to 1 month, pending analysis.

Pharmacokinetic analysis

Gas chromatography with selective nitrogen phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[8] Sample pre-treatment consisted of alkalinized liquid-liquid extraction with ethyl acetate, transfer of the organic extract, evaporation to dryness and subsequent reconstitution in ethyl acetate. This method was validated and proved to be specific, sensitive, accurate (93.3-104.5%) and precise (<5.5%) from 0.192 to 383 µM, with a lower limit of quantification of 0.192 µM for ifosfamide, 2- and 3-dechloroethylifosfamide.
High-performance liquid chromatography was used for determination of 4-hydroxy-ifosfamide pharmacokinetics.[9] In brief this method determined the 4-hydroxyifosfamide-semicarbazone derivative in plasma. The HPLC column used was reversed phase C_8 with acetonitrile-0.025 M potassium dihydrogenphosphate (32:68 v/v) as mobile phase. Detection was performed at 230 nm. Sample pre-treatment consisted of liquid-liquid extraction with ethyl acetate. This method was specific, sensitive, accurate (94.1-107.9%) and precise (<7.2%) from 0.361 to 361 µM, with a lower limit of quantification of 0.361 µM 4-hydroxyifosfamide.

Modelling

Pharmacokinetic model for ifosfamide and its dechloroethylated and hydroxylated metabolites were fitted to the data from the individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling).[10,11] NONMEM estimates population parameters as typical parameter values with their estimated interindividual variability, usually as its S.D., denoted as ω. This is accomplished by allowing each individual's data to be described by subject specific parameters P_i; this is assumed to come from the distribution of parameters in the population according to equation 1.

$$P_i = P_{pop} \times \exp(\eta_i) \qquad \text{(eq. 1)}$$

In which P_{pop} is the parameter value of a typical individual and η is the symmetrically distributed zero-mean variable with the S.D. ω. For mixed effects models, the residual error corresponds to the difference between observed (C_{obs}) and predicted (C_{pred}) concentration by individual parameters (P_i). The residual or intraindividual error was described by a

proportional and additive component according to equation 2,

$$C_{obs} = C_{pred} \times (1+\varepsilon_1) + \varepsilon_2 \quad \text{(eq. 2)}$$

in which ε_1 and ε_2 are zero-mean random variables with S.D.s σ_1 and σ_2. Either component of the residual error could be omitted if it did not provide any improvement of the fit of the data. Model accuracy was evaluated using goodness-of-fit plots and precision of the parameters estimates. In comparison between models the objective function value (which was minus two times the log-likelihood) provided by NONMEM was used. For hierarchical models the difference in objective function value is approximately χ-squared distributed and formal testing between models can be performed, using the log-likelihood ratio test. For non-hierarchical models the objective function value cannot be used for formal testing, but we considered a difference of 9 units between models with identical databases to present a real difference in description of the data.[12] For the graphical goodness-of-fit analysis extensive plotting was available through the use of X-pose, a purpose-built set of subroutines in S-PLUS (Mathsoft, 1997).[13]

A one-compartment model described the change of the amount of ifosfamide (A_{ifo}, µmol) in the central compartment over time by equation 3,

$$\mathbf{t < T_{inf}} : \frac{dA_{ifo}}{dt} = R - \left(CL \times A_{ifo} / V_{ifo} \right)$$

$$\mathbf{t > T_{inf}} : \frac{dA_{ifo}}{dt} = -\left(CL \times A_{ifo} / V_{ifo} \right) \quad \text{(eq. 3)}$$

in which T_{inf} (h) is the infusion duration, R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL (L· h^{-1}· m^{-2}) the ifosfamide clearance over time and V_{ifo} (L· m^{-2}) the volume of distribution. Both CL and V_{ifo} are expressed per body surface area (m^{-2}) in order to accommodate the differences in body size within the paediatric population. The concentration of ifosfamide C_{ifo} (µM) is the ratio of A_{ifo} and V_{ifo}. CL was described with a time dependent increase from an initial clearance (CL_{init}, L· h^{-1}· m^{-2}) by equation 4.

$$CL = CL_{init} + K_{enz} \times t \quad \text{(eq. 4)}$$

In which K_{enz} (L· h^{-2}· m^{-2}) was the enzyme induction rate constant and t (h) the time. No lag-time was used. Boddy et al. described a similar model but with an exponential increase after a lag time from an initial clearance to a maximum clearance.[5]

Ifosfamide and metabolite pharmacokinetics were described simultaneously. The change in the amount of a metabolite (A_m) over time was described by equation 5.

$$\frac{dA_m}{dt} = \left(F_m \times CL \times \frac{A_{ifo}}{V_{ifo}} \right) - \left(K_m \times A_m \right) \quad \text{(eq. 5)}$$

In which K_m (h^{-1}) is the elimination rate constant of the metabolite and F_m is the fraction of ifosfamide metabolized to the metabolite. The values for F_m and the volume of distribution of the metabolite (V_m, L) cannot be estimated separately in this model. Therefore, the ratio of F_m over V_m was estimated: F^* (L^{-1}). The ratio was used to calculate the area under the concentration-time curve (AUC_m) of the metabolite, as described by equation 6.

$$AUC_m = \frac{D}{K_m \times \frac{V_m}{F_m}} \quad \text{(eq. 6)}$$

In which D (mol) was the ifosfamide dose administered to the patient.

The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model according to equation 1. The residual or intraindividual variability of ifosfamide and metabolites kinetics were described separately with a proportional and additive term according to equation 2. The Bayesian estimates of the areas under the concentration-time curves (AUC) of ifosfamide and its metabolites were obtained by describing the cumulative concentrations of these compounds over a given time. AUCs were obtained with extrapolation of ifosfamide and metabolite concentration-time profiles to 48 hours after the last ifosfamide infusion, where concentrations were below the LLQ.

Validation

In order to assess objectively the predictive capability of our model a cross-validation was performed using a bootstrap technique.[14] The patients were divided in 5 groups: 3 groups with 6 and 2 with 7 patients. The pharmacokinetic model was fitted to the data from 4 of the 5 groups and parameter estimates were obtained. These parameter estimates were then used to generate concentration predictions for the remaining patients in the fifth group. This process was repeated four more times so that predictions could be generated for all 32 patients.

Results

Pharmacokinetic assessment resulted in 133 ifosfamide, 106 2-dechloroethylifosfamide, 116 3-dechloroethylifosfamide and 76 4-hydroxyifosfamide observed concentrations, as represented in figure 2. The sparse data approach model was able to estimate population values for the pharmacokinetic parameter of ifosfamide and its dechloroethylated and hydroxylated metabolites (table 1). The pharmacokinetic model also estimated the differences between patients (interindividual variability) and within the patient (intraindividual variability). Table 1 also contains the accuracy of parameter estimation as described by the relative error. The relationship between the model predictions, Bayesian predictions, cross-validated predictions and the observed ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide concentrations are represented in figure 3. In general, no trends or extreme outlyers were observed, although one patient evidently had different 4hydroxyifosfamide concentrations of about 10 µM.

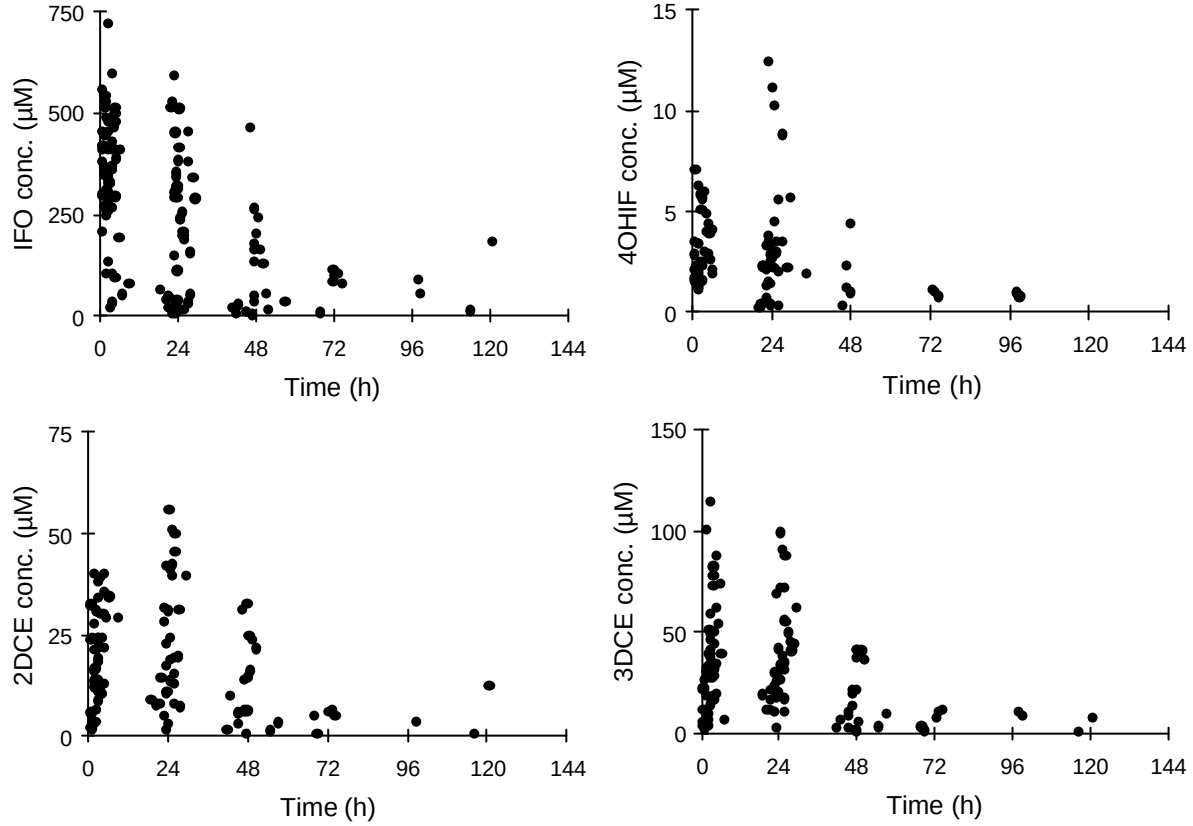

Figure 2. *Plots of all observed ifosfamide, 2-dechloroethylifosfamide (2DCE), 3-dechloroethyl-ifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) concentrations over time.*

Table 1. *Estimates of pharmacokinetic population parameters for ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) with their relative standard error of the mean, interindividual variability and residual variability.*

Parameter	Mean	Rel. err.	I.I.V.	R.V.	Mean
IFO					
CL_{init} ($L \cdot h^{-1} \cdot m^{-2}$)	2.36	14%	43%	P.E. IFO	48%
V_{ifo} ($L \cdot m^{-2}$)	20.6	8%	32%	A.E. IFO (µM)	3.18
K_{enz} ($L \cdot h^{-2} \cdot m^{-2}$)	0.0493	21%			
2DCE					
F^* (L^{-1})	0.0976	57%	23%	P.E. 2DCE	41%
K (h^{-1})	3.64	56%		A.E. 2DCE (µM)	2.24
3DCE					
F^* (L^{-1})	0.0328	31%	34%	P.E. 3DCE	40%
K (h^{-1})	0.445	39%		A.E. 3DCE (µM)	1.98
4OHIF					
F^* (L^{-1})	0.0230	36%	53%	P.E. 4OHIF	47%
K (h^{-1})	7.67	37%		A.E. 4OHIF (µM)	0.448

Rel. Err.=Relative error of the mean, I.I.V.=interindividual variability, R.V.=residual variability, CL_{init}=initial ifosfamide clearance, V_{ifo}=volume of distribution of ifosfamide, K_{enz}=autoinduction formation rate, F^*=ratio of fraction metabolized and volume of distribution of metabolite, K=first-order rate constant for metabolite

elimination, P.E.=proportional intraindividual error, A.E.=additive intraindividual error.

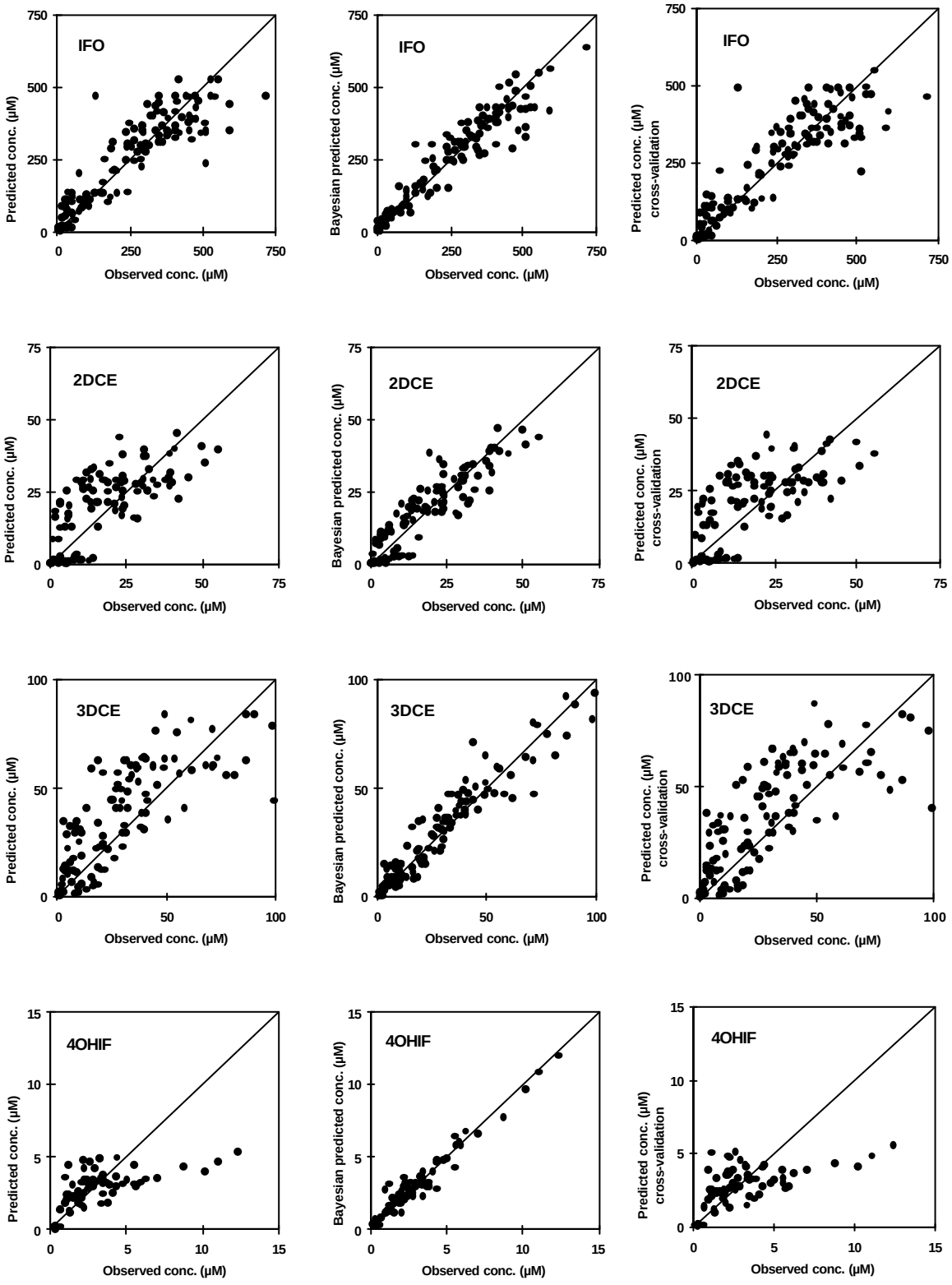

Figure 3. *The relationships between model predictions (left panel), individual Bayesian estimates (middle panel), cross-validated predictions (right panel) and observed ifosfamide, 2-dechloroethyl-ifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) concentrations.*

The relationship between the ifosfamide clearance and time is represented in figure 4. Modest interindividual variability is observed in clearance. The relationships between the area under the concentration-time curve (AUC) of ifosfamide, 2- and 3-dechloroethyl-ifosfamide and 4-hydroxyifosfamide and the ifosfamide dose administered are represented in figure 5. The AUC is a measure of exposure to the compound.

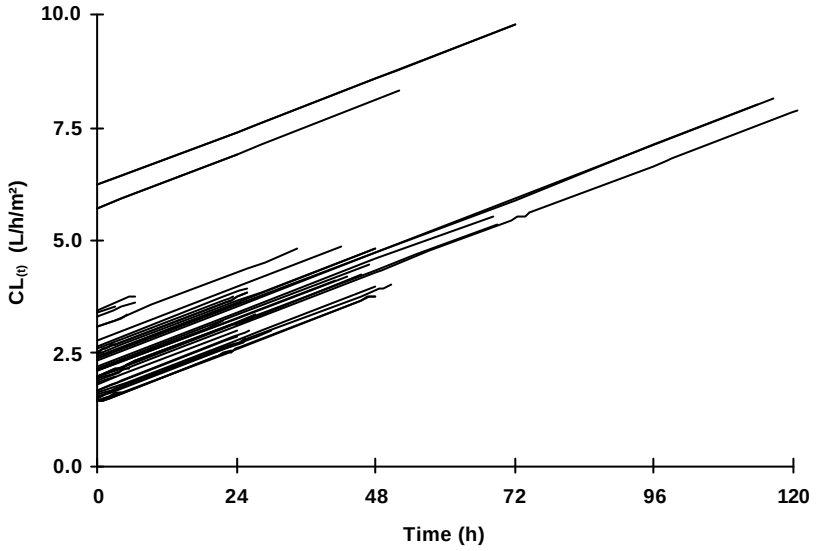

Figure 4. *The relationship between the ifosfamide clearance ($CL_{(t)}$, L/h/m²) and the time for all individuals. The profiles were obtained on basis of the individual Bayesian parameter estimates provided by NONMEM.*

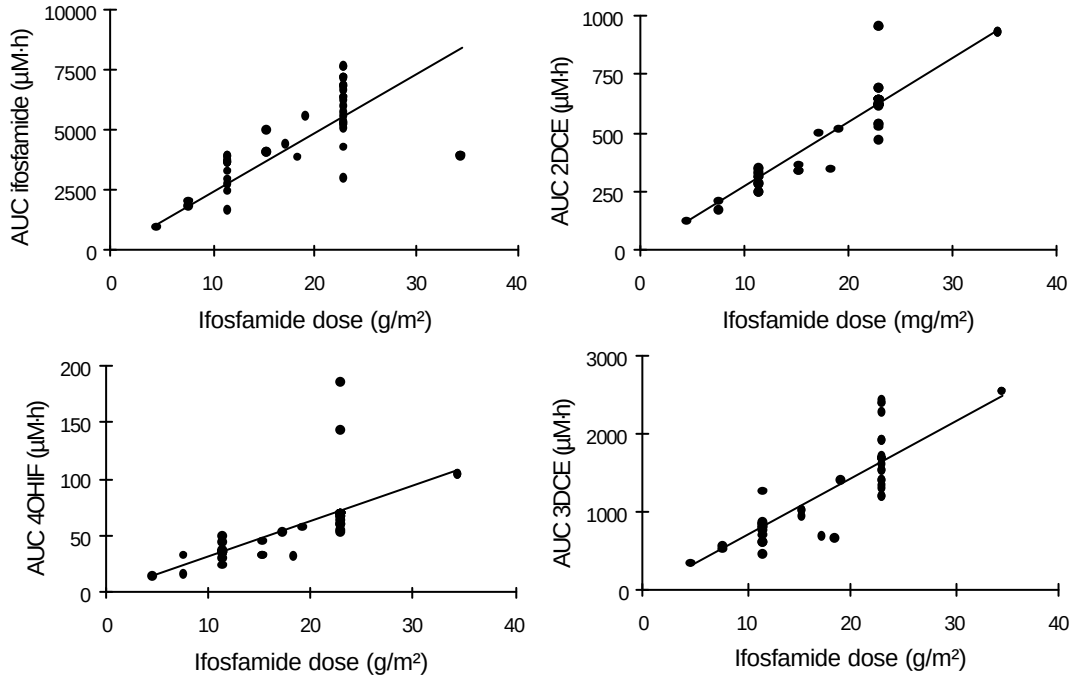

Figure 5. *The relationship between the ifosfamide dose (g/m²) and ifosfamide, 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) exposures as calculated by the area under the concentration-time curve (AUC) of each patient.*

Discussion

Only limited information is available on the pharmacokinetics of ifosfamide and its metabolites in paediatric patients.[15] The lack of adequate pharmacokinetic research can be explained by both the lack of reliable and sensitive bioanalytical assays for ifosfamide and its metabolites and due to the problems involving paediatric studies. Paediatric studies have always been plagued by ethical issues. Due to these concerns drugs used in paediatric population have been less assessed compared to those used in adult populations. Especially pharmacokinetic studies are difficult to execute, because children and their parents may find blood sampling very distressing. It is therefore essential that the number of blood samples is kept to the minimum required. An opportunistic limited sampling approach only allows assessment of fairly straightforward pharmacokinetics and excludes for instance multi-compartment modelling. A solution to this problem is a population approach. A population approach pools all observed concentrations in a population of patients, yielding population values for the pharmacokinetic parameters. Bayesian estimates are then used to calculate individual pharmacokinetic parameters. Nevertheless, a reduced number of samples will result in an increase in the minimum number of patients required.[16] An additional problem in young children relates to the volume of blood sample collected, necessitating the use and development of micro-assays.

A minimal impact on the quality of life of the children was obtained by applying a sparse data approach, which limited the pharmacokinetic assessment to routine phlebotomies and indwelling catheters. This approach allowed pharmacokinetic assessment of all parameters for each individual. In contrast, conventional non-population based methods are not able to estimate these parameters based only on one or two observations. The Bayesian estimation step was based on the information obtained from the sparse individual observations supplemented with information from the population values. The model is adequate in describing the ifosfamide and metabolite concentration-time (profiles including autoinduction) in this paediatric population (figure 3). The description of these concentration-time profiles enabled calculation of the exposures to these compounds (figure 5).

During the development of the model a comparison was made between a pharmacokinetic model with time-dependent autoinduction and a model with ifosfamide concentration-dependent autoinduction.[5,17] The sparse data necessitated the use of a simplified model with a linear increase of clearance over time with time-dependent autoinduction.

Boddy et al. also described the pharmacokinetics of ifosfamide and its metabolites after 3 g/m² ifosfamide in one-hour infusions for three consecutive days.[3] Their model did not include autoinduction, but rather the pharmacokinetics were estimated separately each consecutive day. They observed the clearance of ifosfamide to increase from 3.81±1.08 to 6.34±1.81 $L \cdot h^{-1} \cdot m^{-2}$ over three days with a volume of distribution of 21.8±6.5 $L \cdot m^{-2}$, based on an average body surface area of 0.82 m^2 and weight of 22.1 kg. Our model resulted in similar values (figure 4 and table 1). The initial CL ranged from 1.40 to 6.20 and increased maximally to 9.75 $L \cdot h^{-1} \cdot m^{-2}$. The volume of distribution ranged between 8.73 and 33.5 $L \cdot m^{-2}$.

The sparse data were not sufficient to estimate possible interindividual differences in the enzyme induction rate K_{enz}. A similar pharmacokinetic model without estimation of

autoinduction was also tested. The inclusion of the K_{enz} in the model, however, resulted in an improved goodness-of-fit as calculated by the objective function of the model. The objective function increased significantly from 3035 to 3056. The exclusion of K_{enz} resulted in an increase of CL_{init}, but also decreased the accuracy of the estimation of the metabolite parameters. The relative errors of the metabolite parameters without estimation of K_{enz} ranged from 51 to 91%.

Besides simultaneous estimation ('one-step approach') of the pharmacokinetic parameters of ifosfamide and its metabolites, a 'two-step approach' was also investigated. This 'two-step approach' estimated the individual pharmacokinetic parameters of ifosfamide first, independently from the metabolites. Then these individual estimations were fixed in a second estimation step of the metabolite pharmacokinetics. The objective function of the two approaches cannot be compared, because the models use to describe the pharmacokinetics of ifosfamide and metabolites were not hierarchical. The 'two-step approach' estimated a relatively low residual proportional variability for ifosfamide (27.1%), but the additive component was higher with 39.8 µM. Furthermore, the relative errors of all parameters were higher with the two-step approach. The simultaneous approach resulted in more precise estimates, because then the metabolites had an influence on the estimation of the pharmacokinetics of the parent-compound. This resulted in optimal use of all information in this sparse data approach.

The cross-validation was performed using the predicted concentrations and not the individual Bayesian predictions, because of the large differences in number of samples of each patient. Normally, bootstrapping-techniques are repeated more than 200 times, but due to extensive calculation-time this was not feasible.[14] The residual error model of ifosfamide and those of the metabolites assume a larger imprecision with lower concentrations. This was modelled with the additive and proportional residual error. Thus no bias or precision could be calculated, because this would not take into account the different precisions per concentration. The best indication of lack of bias is seen in the scatterplot of figure 3 when comparing the left with the right panel.

The ifosfamide dose and the ifosfamide and metabolite exposures (AUC) exhibit linear relationships (figure 5). Modest interpatient differences in exposure are observed. Since ifosfamide was typically given as short duration (1-3 h) infusions no interindividual differences in effect of autoinduction on the metabolic routes were expected. This explains also why no interindividual variability in autoinduction could be estimated in this population.

The sparse data approach for describing the pharmacokinetics of ifosfamide and its metabolites can be used to investigate the pharmacokinetic-pharmacodynamic relationships of the specific metabolites with the efficacy and toxicities of ifosfamide treatment in children, based on opportunistic sampling. These pharmacokinetic-pharmacodynamic relationships can assist in improving ifosfamide treatment in a paediatric setting, tailoring the treatment to the specific needs of the individual child.

In conclusion, the application of a sparse data approach for the determination of the population pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide in a paediatric population was feasible. A bootstrap technique demonstrated the validity of the population pharmacokinetic model.

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Chapter 4.3

Modulation of the cytochrome P450-mediated metabolism of ifosfamide by ketoconazole and rifampicin

Summary

The autoinducible metabolic transformation of the anti-cancer agent ifosfamide involves activation through 4-hydroxyifosfamide to the ultimate cytotoxic ifosforamide mustard and deactivation to 2- and 3-dechloroethylifosfamide with concomitant release of the neurotoxic chloroacetaldehyde. Activation is mediated by the cytochrome P450-3A4 (CYP3A4) and deactivation by CYP3A4 and 2B6. The aim of this study was to investigate modulation of the CYP-mediated metabolism of ifosfamide with ketoconazole and rifampicin, a potent inhibitor of CYP3A4 and inducer of CYP3A4/2B6, respectively. In a double randomized, two-way cross-over study a total of 16 patients received 3 g/m²/24 hours ifosfamide i.v., either alone or in combination with 200 mg ketoconazole bid (1 day pre-treatment and 3 days concomitant administration) or 300 mg rifampicin bid (3 days pre-treatment and 3 days concomitant administration). Plasma pharmacokinetics and urinary excretion of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxy-ifosfamide were assessed in both courses. Data analysis was performed with a population pharmacokinetic model with description of autoinduction of ifosfamide. Rifampicin increased the clearance of ifosfamide at the start of therapy with 102%. The fraction of ifosfamide metabolized to the dechloroethylated metabolites was increased, whereas exposure to the metabolites was decreased due to increased elimination. The fraction metabolized and exposure to 4-hydroxyifosfamide were not significantly influenced. Ketoconazole did not affect the fraction metabolized or exposure to the dechloroethylated metabolites, whereas both parameters were reduced with 4-hydroxyifosfamide. As a result co-administration of ifosfamide with ketoconazole or rifampicin did not produce changes in the pharmacokinetics of the parent or metabolites that may result in increased benefit of ifosfamide therapy.

Introduction

Chapter 4.3

Ifosfamide (Holoxan®, Ifex®) is an alkylating agent used as a single chemotherapeutic or in combination schedules. It has been proven to be active against a number of solid tumours and haematological malignancies in adults and children.[1] Ifosfamide is a prodrug, which needs activation by cytochrome P450-3A4 (CYP3A4) to form 4-hydroxyifosfamide. Spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard and acrolein.[1] The alkylating activity of ifosforamide mustard is both responsible for the anti-tumour activity and haematological toxicity. Acrolein causes haemorrhagic cystitis, which can generally be prevented by sodium-2-mercaptoethanesulfonate (MESNA) co-administration.[1] Ifosfamide is also deactivated by CYP3A4 and possibly CYP2B6 to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide. Dechloroethylation, however, yields an equimolar amount of chloroacetaldehyde, which is held responsible for neurotoxicity observed in about 10% of all patients receiving conventional single-agent dosing of ifosfamide.[2] Renal tubular abnormalities have been observed as well and may also be correlated with the extent of metabolic formation of chloroacetaldehyde. Chloroacetaldehyde is very unstable and therefore, assessment of 2- and 3-dechloroethylifosfamide is preferred when quantifying the relationship between the pharmacokinetics and neurotoxicity after ifosfamide treatment. Ifosfamide metabolism is subject to autoinduction, which leads to an increase in metabolism of ifosfamide over time.[1] Both activation and deactivation routes are mainly mediated by CYP3A4, making this isoenzyme a suitable target for development of specific inhibition or hetero-induction regimens and thereby directing ifosfamide metabolism to a more favourable toxicity-efficacy profile: minimized risk of neurotoxicity (deactivation) and maximized anti-tumour activity (activation) of ifosfamide treatment.[3]

Ketoconazole is a broad spectrum anti-fungal agent. It has been shown to reduce the metabolism of other drugs sharing the CYP3A4 pathway.[4] The *in vitro* inhibition constants (K_i) for ketoconazole towards CYP3A4 index substrates are usually in the nanomolar range.[4] Therapeutic concentrations of ketoconazole significantly inhibited the formation of N-dechloroethylated metabolites of ifosfamide by human liver microsomes *in vitro*.[5] Rifampicin (rifampin) is a semi-synthetic macrocyclic antibiotic and a highly potent, albeit non-specific, inducer of CYP.[6] Induction is achieved through activation of de novo synthesis of CYP and typically requires several days to develop.[7] In a recent *in vitro* study it was demonstrated that rifampicin was capable of inducing ifosfamide metabolism in cultured human hepatocytes.[8] Hydroxylation of ifosfamide was increased 200-400% and CYP2B6, 2C8, 2C9 and 3A4 levels were elevated in the cell culture.

The aim of this study was to investigate modulation of the CYP-mediated metabolism of ifosfamide by ketoconazole a potent inhibitor of CYP3A4 and rifampicin a potent inducer of CYP. The activation and deactivation routes of ifosfamide were monitored using the pharmacokinetic profiles of 4-hydroxyifosfamide and 2- and 3-dechloroethyl-ifosfamide, respectively.

Methods

Patients

Eligible patients had incurable malignancies, no major organ disturbances, no active infection, no major medical illness or signs of CNS involvement or leptomeningal disease, a life expectancy of 3 months or more and were at least 6 and 4 weeks chemotherapy- and radiotherapy-free, respectively. Other eligibility criteria were: age between 18 and 75 years, adequate contraception, white blood cells 3.5 x 10^9/L and platelets 100 x 10^9/L, serum bilirubin 25 µmol/L, serum creatinine 125 µmol/L and creatinine clearance 60 ml/min, serum ASAT 50 U/L and serum ALAT 60 U/L. The study protocol was approved by the Ethical Board of the University Hospital of Maastricht. The study was conducted at the department of Internal Medicine of the above mentioned hospital between April 1998 and April 2000. All patients gave written informed consent.

Study design

The study was designed as a double randomized, cross-over study. Sixteen patients received two courses of palliative treatment with ifosfamide, at a three week interval. Ifosfamide (Holoxan®, ASTA Medica B.V., Diemen, The Netherlands) was administered at 3 g/m² by means of 24-hour continuous intravenous (i.v.) infusion. No dose reductions were allowed, but the second course could be delayed by one week if the eligibility criteria for the white blood cells and platelets counts were not met.
Eight patients were randomized to the co-administration of twice daily 200 mg ketoconazole orally for four days starting one day prior to the ifosfamide infusion. Four patients received ketoconazole during their first course of ifosfamide treatment and four patients received ketoconazole during their second course of ifosfamide treatment. The other eight patients were randomized to the co-administration of twice daily 300 mg rifampicin orally for 6 days, starting 3 days prior to the ifosfamide infusion. Four patients received rifampicin during their first course of ifosfamide treatment and four patients received rifampicin during their second course of ifosfamide treatment.
Supportive care consisted of MESNA (Uromitexan®, ASTA Medica B.V., Diemen, The Netherlands) at 0.75 g/m² in 1 hour prior to the ifosfamide infusion. This was followed by 1.5 g/m² mesna in 24 hours during the ifosfamide infusion and 0.75 g/m² mesna in 12 hours thereafter. Extensive hydration to prevent haemorrhagic cystitis was achieved by administration of 500 ml 0.9% NaCl in 1 hour prior to the ifosfamide infusion, 3000 ml 0.9% NaCl in 24 hours during the ifosfamide infusion and 2000 ml 0.9% NaCl in 24 hours after the ifosfamide infusion. Eight mg of the anti-emetic ondansetron was given intravenously every eight hours during the ifosfamide infusion. No use of dexamethasone, barbiturates, cimetidine, macrolide antibiotics and lorazepam was allowed, because of possible CYP3A4 interactions.

Pharmacokinetic sampling

Blood samples were drawn from an indwelling intravenous cannula placed in the arm contralateral to the arm receiving ifosfamide prior to the start of the ifosfamide infusion and 6, 12, 18, 24, 25, 27, 30, 36 and 48 hours thereafter. A 10 ml volume of whole blood was

collected in a lithium heparin-coated Vacutainer® (Becton-Dickinson, Plymouth, UK) and placed on ice water. The plasma was immediately separated by centrifugation at 1,000 g for 5 min at 4°C. The plasma was aliquoted in three volumes of which two were precisely 1-ml volumes. To these two 1-ml volumes, 100 µl 2 M semicarbazide solution at pH 7.40 were added to stabilize 4-hydroxyifosfamide. The remaining plasma was used for ifosfamide, 2- and 3-dechloroethylifosfamide analysis. Urine was collected for up to 72 hours after the start of the ifosfamide infusion and an aliquot was analysed for ifosfamide, 2- and 3-dechloroethylifosfamide. No 4-hydroxyifosfamide can be detected in urine due to rapid degradation. Both plasma and urine samples were stored at -70°C, pending analysis.

Bioanalysis

Gas chromatography with selective nitrogen-phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[9] Sample pre-treatment consisted of alkalinized liquid-liquid extraction with ethyl acetate, transfer of the organic extract, evaporation to dryness and subsequent reconstitution in ethyl acetate. This method was validated and proved to be specific, sensitive, accurate (93.3-104.5%) and precise (within- and between-day precision <5.5%) within the concentration range of 0.192 to 383 µM, with a lower limit of quantification (LLQ) of 0.192 µM for ifosfamide, 2- and 3-dechloroethylifosfamide.
High-performance liquid chromatography (HPLC) was used for the determination of 4-hydroxyifosfamide plasma concentrations.[10] In brief, this method determined the 4-hydroxyifosfamide-semicarbazone derivative in plasma. Sample pre-treatment consisted of liquid-liquid extraction with ethyl acetate. The HPLC column used was a reversed phase C_8 column with acetonitrile-0.025 M potassium dihydrogenphosphate (32:68 v/v) as mobile phase. Detection was performed at 230 nm. This method was specific, sensitive, accurate (94.1-107.9%) and precise (within- and between-day precision <7.2%) within the concentration range of 0.361 to 361 µM, with a LLQ of 0.361 µM 4-hydroxyifosfamide.

Data evaluation

Pharmacokinetic models were fitted to the data from the sixteen individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling, version V 1.1, double precision, first order estimation).[11,12]

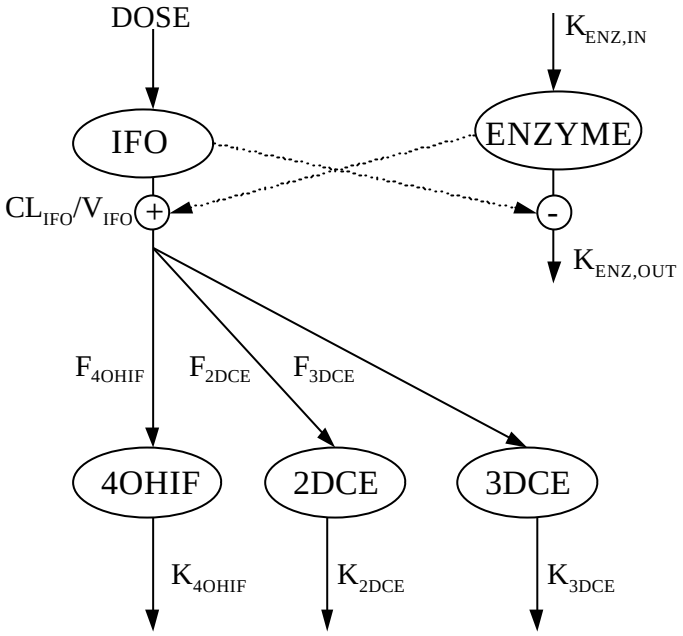

Figure 1. *Pharmacokinetic model for ifosfamide metabolism describing the autoinducible pharmacokinetics of ifosfamide (IFO) and the pharmacokinetics of 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM). Autoinduction is modelled with a hypothetical enzyme compartment described by an enzyme formation rate ($K_{enz,in}$) and elimination rate ($K_{enz,out}$). At t=0 $K_{enz,in}$ equalled $K_{enz,out}$. A higher amount of enzyme increases the clearance of IFO (CL_{IFO}). The amount of IFO described by the IFO concentration and IFO volume of distribution (V_{IFO}), inhibits $K_{enz,out}$. Metabolite compartments are described by fraction of ifosfamide metabolized to the metabolite (F) and elimination rates (K).*

The non-linear pharmacokinetics of ifosfamide were described using a recently developed model (figure 1), which incorporated the development of autoinduction.[13,14] According to these recent studies the time-dependent pharmacokinetics of ifosfamide can be described by a one-compartment model. The change of the amount of ifosfamide (A_{ifo}, µmol) in the central compartment over time can be described by equation 1,

$$\mathbf{t < T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = R - \left(CL \times A_{ifo} \Big/ V_{ifo}\right) \text{ and}$$

$$\mathbf{t > T_{inf}}\ :\ \frac{dA_{ifo}}{dt} = -\left(CL \times A_{ifo} \Big/ V_{ifo}\right) \qquad \text{(eq. 1)}$$

in which T_{inf} (h) is the infusion duration, R (µmol· h^{-1}) is the infusion rate of ifosfamide, CL (L· h^{-1}) the ifosfamide clearance, which changes over time and V_{ifo} (L) the volume of distribution. The concentration of ifosfamide C_{ifo} (µM) is the ratio of A_{ifo} and V_{ifo}. In turn, autoinduction can be described using a hypothetical enzyme compartment. CL can then be expressed by an initial clearance ($CL_{ini,ifo}$, L· h^{-1}) multiplied by the relative amount of enzyme (A_{enz}) in the hypothetical enzyme compartment, as given in equation 2.

$$CL = CL_{ini,ifo} \times A_{enz} \qquad \text{(eq. 2)}$$

The change of A_{enz} (no dimension) over time in the enzyme compartment is dependent on C_{ifo} as follows,

$$\frac{dA_{enz}}{dt} = K_{enz,out} - K_{enz,out} \times A_{enz} \times \left(1 - \frac{C_{ifo}}{C_{ifo} + IC_{50}}\right) \quad \text{(eq. 3)}$$

in which $K_{enz,out}$ (h^{-1}) is the first-order rate constant for enzyme degradation/inactivation and IC_{50} (µM) is the ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation. The induction half-life of the enzyme ($T_{½enz}$, h) was calculated by the ratio of ln(2) and $K_{enz,out}$.

The pharmacokinetics of ifosfamide and metabolites were described sequentially: Bayesian estimations (posthocs) of the pharmacokinetic parameters of ifosfamide were used for the description of the pharmacokinetics of the metabolites. The change in the amount of a metabolite (A_m) over time could be described by equation 4.

$$\frac{dA_m}{dt} = \left(F_m \times CL \times \frac{A_{ifo}}{V_{ifo}}\right) - \left(K_m \times A_m\right) \quad \text{(eq. 4)}$$

in which K_m (h^{-1}) is the elimination rate constant of the metabolite and F_m is the fraction of ifosfamide metabolized to the metabolite. The values for F_m and the volume of distribution of the metabolite (V_m, L) cannot be estimated separately in this model. Therefore, the ratio of F_m over V_m was estimated: F^* (L^{-1}).

The residual or intraindividual variability of ifosfamide and metabolites were described separately with a proportional and additive term. The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model.

The posthocs of the areas under the concentration-time curve (AUC) of ifosfamide were obtained by describing the cumulative concentrations of these compounds over a given time. AUCs of ifosfamide were obtained by extrapolation of concentration-time profiles to 144 hours after the start of the infusion, where ifosfamide concentrations were below the LLQ. The AUCs of the metabolites were calculated using equation 5,

$$AUC_m = \frac{D \times F^*}{K_m} \quad \text{(eq. 5)}$$

in which D represents the ifosfamide dose (µmol).

The effect of ketoconazole and rifampicin on the pharmacokinetics of ifosfamide, 2-dechloroethylifosfamide, 3-dechloroethylifosfamide and 4-hydroxyifosfamide was estimated simultaneously, as described by equation 6.

$$P_{pop} = \theta_{control} \times \left(\theta_{ketoconazole} + \theta_{rifampicin} + 1\right) \quad \text{(eq. 6)}$$

in which P_{pop} is the population pharmacokinetic parameter, $\theta_{control}$ is the estimate for this parameter without modulation, $\theta_{ketoconazole}$ is the change in this parameter due to ketoconazole co-administration and $\theta_{rifampicin}$ is the change in this parameter due to rifampicin co-administration. Significant differences due to modulation were tested by calculating the 95%-confidence intervals of $\theta_{ketoconazole}$ and $\theta_{rifampicin}$ using the relative error of the respective mean. If the 95%-confidence interval did not include zero, the observed difference was significant ($p<0.05$). The percentage change in AUCs of the metabolites was also tested according to equation 6 after reparametrization of the model according to equation 5. A non-parametric test (Kolmogorov-Smirnov test for two independent samples) was conducted to

determine significant differences in AUC of ifosfamide with a modulator compared to control. The urinary excretions (UE) for ifosfamide and 2- and 3-dechloroethylifosfamide were calculated based on the equimolar amount of the dose administered. Differences in urinary excretion were tested with a paired samples t-test using SPSS software (Version 6.1 for Windows; SPSS, Inc., Chicago, IL, USA).

Results

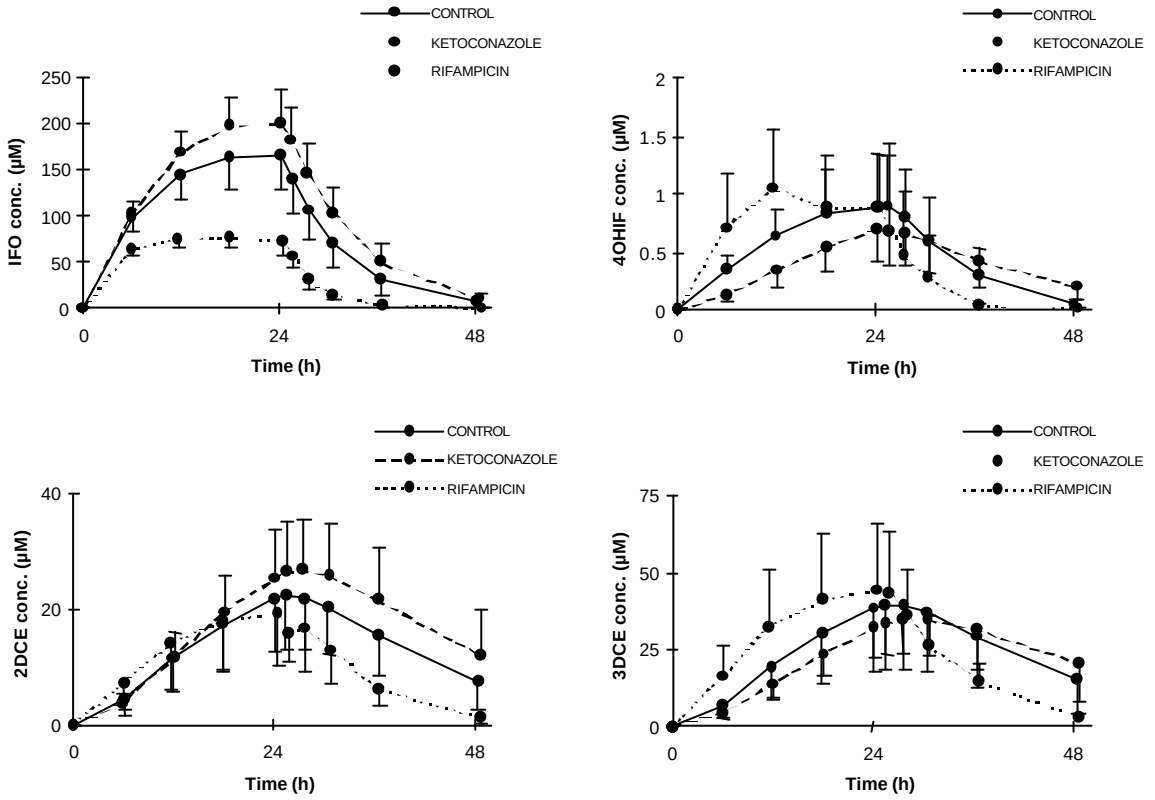

Figure 2. *Mean (±SD) observed concentration-time profiles of ifosfamide (IFO), 2-dechloroethyl-ifosfamide (2DCE), 3dechloroethylifosfamide (3DCE) and 4hydroxyifosfamide (4OHIF) in 16 patients receiving 3 g/m²/24 hours ifosfamide i.v., either alone or in combination with 200 mg ketoconazole bid (one day pre-treatment and three days concomitant administration) or 300 mg rifampicin bid (three days pre-treatment and three days concomitant administration).*

All 16 patients were evaluable for the determination of the plasma pharmacokinetics of ifosfamide and its metabolites and their excretion in urine. The mean observed ifosfamide and metabolite concentration-time profiles are shown in figure 2. The pharmacokinetic parameters of ifosfamide and its metabolites and changes due to CYP modulation with ketoconazole and rifampicin are presented in table 1.

The pharmacokinetic data contained insufficient information to estimate the interindividual variability on the enzyme induction parameters $K_{enz,out}$ and IC_{50}. The influence of modulation on these parameters could therefore not be evaluated. Autoinduction of ifosfamide developed with an average value for $T_{½,enz}$ ($\ln(2)/K_{enz,out}$) of 25.9 h. It was assumed that the volumes of distribution of the ifosfamide and the metabolites were not affected by the modulation. The

changes in exposure to ifosfamide and its metabolites due to modulation are depicted in figure 3.

Table 1. *Pharmacokinetic parameters of ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) and changes (%) after CYP3A4-modulation with ketoconazole (n=8) or rifampicin (n=8) in 16 patients receiving 3 g/m²/24 hours ifosfamide i.v..*

Parameter	Control Mean±sem	I.I.V.	Ketoconazole Mean change	significance	Rifampicin Mean change	significance
$CL_{ini,ifo}$ (L· h^{-1})	3.44±0.33	20%	-11%	p<0.05	+102%	p<0.05
V_{ifo} (L)	42.7±2.52	19%				
$K_{enz,out}$ (h^{-1})	0.0268±0.0056					
IC_{50} (µM)	13.2±9.8					
AUC_{ifo} (µM· h)	5263±947		+14%	ns	-49%	p<0.05
UE_{ifo}	17%	47%	+26%	ns	-43%	p<0.05
F^*_{2dce} (L^{-1})	0.00241±0.00043	83%	+17%	p<0.05	+23%	p<0.05
K_{2dce} (h^{-1})	0.131±0.092	150%	-5%	ns	+82%	p<0.05
AUC_{2dce} (µM· h)	867±92		+23%	p<0.05	-32%	p<0.05
UE_{2dce}	7%	40%	+39%	ns	+17%	ns
F^*_{3dce} (L^{-1})	0.00469±0.00075	61%	-10%	ns	+15%	ns
K_{3dce} (h^{-1})	0.0475±0.0128	52%	-4%	ns	+320%	p<0.05
AUC_{3dce} (µM· h)	1950±245		-6%	ns	-72%	p<0.05
UE_{3dce}	8%	30%	-3%	ns	+11%	ns
F^*_{4ohif} (L^{-1})	0.000295±0.000062	42%	-48%	p<0.05	+1200%	ns
K_{4ohif} (h^{-1})	0.179±0.068	33%	-26%	ns	+1370%	ns
AUC_{4ohif} (µM· h)	32±4		-30%	ns	-11%	ns

I.I.V.=interindividual variability, $CL_{ini,ifo}$=initial ifosfamide clearance, V_{ifo}=volume of distribution of ifosfamide, $K_{enz,out}$=autoinduction formation rate, IC_{50}=ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation, F^*=ratio of fraction metabolized and volume of distribution of metabolite, K=first-order rate constant for metabolite elimination, AUC=area under the concentration-time curve, UE=urinary excretion, ns=not significant.

Table 1 demonstrates that $CL_{ini,ifo}$ was decreased by ketoconazole. Concomitant increases in AUC_{ifo} (figure 3) and UE_{ifo} were observed although not significantly different from the control values. When assuming that the volume of distribution of the metabolites is not affected by concomitant administration of the modulators, it can be concluded that ketoconazole significantly increased the fraction of ifosfamide metabolized to 2-dechloro-ethylifosfamide (F^*_{2dce}), whereas the elimination rate of 2-dechloroethylifosfamide (K_{2dce}) was not changed. AUC_{2dce} and UE_{2dce} were slightly increased although the latter not significantly (figure 3, table 1). No changes in F^*, K, AUC and UE were observed for 3-dechloroethylifosfamide. For 4-hydroxyifosfamide decreased values for AUC, K and F^* were observed with the latter reaching statistical significance.

$CL_{ini,ifo}$ was doubled by rifampicin, resulting in a decreased AUC_{ifo} and UE_{ifo}. A significant increase in F^*_{2dce} and K_{2dce} was observed with the inducer, with the increase in elimination being the most pronounced (82%). Consequently, the AUC_{2dce} decreased with rifampicin (figure 3). The decrease of AUC_{2dce} did not, however, affect UE_{2dce}, which increased slightly. Similar results were observed for 3-dechloroethylifosfamide. Both F^*_{3dce} and K_{3dce} increased; the increase of the latter was significant. This resulted in a decrease of AUC_{3dce} whereas UE_{3dce} was not influenced. The changes in F^*_{4ohif} and K_{4ohif} by rifampicin were 12-fold and 14-fold, respectively. These changes were, however, not significant. The changes in F^*_{4ohif} and K_{4ohif} were comparable; the AUC_{4ohif} changed therefore only slightly. It should be noted that the estimates of F^*_{4ohif} and K_{4ohif} were highly correlated.

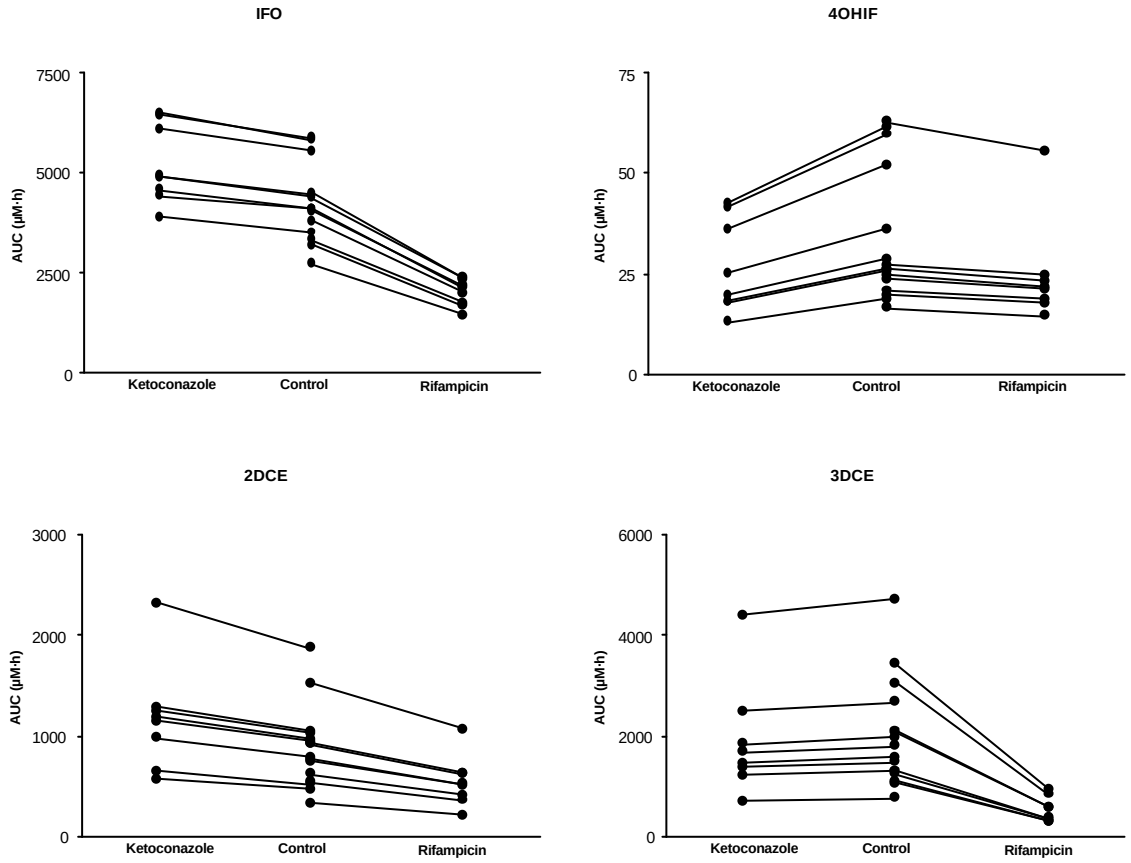

Figure 3. *Bayesian estimation of the areas under the concentration-time curves (AUC) of ifosfamide (IFO), 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE) and 4-hydroxyifosfamide (4OHIF) in 16 patients receiving 3 g/m²/24 hours ifosfamide i.v., either alone or in combination with 200 mg ketoconazole bid (one day pre-treatment and three days concomitant administration) or 300 mg rifampicin bid (three days pre-treatment and three days concomitant administration).*

Discussion

Although drug-drug interactions are generally avoided in pharmacotherapy, CYP interactions have also been used to increase therapeutic efficacy. For instance, the anti-retroviral drug ritonavir, an inhibitor of CYP3A4, is used to enhance efficacy of saquinavir anti-retroviral therapy by increasing the AUC of saquinavir 58-fold.[15] Cyclosporin is used to increase the

oral uptake of paclitaxel by CYP and P-glycoprotein interaction.[16] Modulation of ifosfamide metabolism may be used to decrease formation of the neurotoxic metabolite chloracetaldehyde and to increase exposure to 4-hydroxy-ifosfamide, which is a marker for anti-tumour activity. Modulation of the metabolism of ifosfamide in rats has been demonstrated with phenobarbital and dexamethasone, which are potent inducers of CYP2B and 3A in rats, respectively, and metyrapone, which is a potent inhibitor of CYP2B1 in rats.[17] Modulation of ifosfamide metabolism in humans has not yet been successful. In a study by Lokiec et al. modulation was not detected when ifosfamide was administered together with the inducer phenobarbital.[18] This failure could, however, be explained by the fact that enzyme induction by barbiturates typically requires several days to develop.[7] Therefore, the simultaneous administration of phenobarbital was not likely to result in hetero-induction of ifosfamide.

In this study modulation of the CYP-mediated metabolism of ifosfamide was investigated by co-administration of ketoconazole and rifampicin in a double randomized, cross-over study in 16 patients. In conventional schemes ifosfamide is administered as a continuous 24-hour infusion with a dosage of 5 g/m².[1] In this study the dosage was decreased to 3 g/m² to prevent possible unacceptable neurotoxicity as a result from the co-administration of the modulators. A non-linear pharmacokinetic population model described the autoinducible pharmacokinetics of ifosfamide, 2- and 3-dechloroethyl-ifosfamide and 4-hydroxyifosfamide adequately. In this study conventional non-compartmental analysis of individual plasma concentration-time profiles did not allow accurate estimation of the metabolite elimination rates in all patients, due to insufficient data to accurately extrapolate the profiles to infinity. This was overcome by using a population pharmacokinetic approach, which allows the estimation of pharmacokinetic parameters when data are sparse.[19]

In this study no ketoconazole or rifampicin concentrations were determined, but literature data suggested that co-administration at these dosages should have resulted in significant inhibition and induction of CYP3A4, respectively. Tsunoda et al. reported that after 200 mg ketoconazole orally twice daily for two days, plasma concentrations ranged between 0.15 and 9.13 µM, which was well above the K_i of 0.01µM, thus resulting in complete inhibition of CYP3A4-mediated midazolam metabolism.[20] Administration of 300 mg rifampicin bid if given for at least 3 days, has been reported to produce CYP3A4 up-regulation in various clinical studies.[6]

In our study ketoconazole decreased ifosfamide metabolism, as indicated by the decreased clearance and increased urinary excretion (table 1). Furthermore, it slightly decreased activation, as indicated by the decreased F^*_{4ohif} and AUC_{4ohif}, and did not affect deactivation to 3-dechloroethylifosfamide. F^*_{2dce} and AUC_{2dce} were slightly increased, which resulted in more urinary excretion (UE_{2dce}). The latter difference was, however, not significant due to interindividual variability. Overall, only marginal influence of ketoconazole on exposure to 4-hydroxyifosfamide and the dechloroethylated metabolites was observed. Rifampicin increased ifosfamide metabolism as indicated by an increased $CL_{ini,ifo}$ and decreased AUC_{ifo} and UE_{ifo}. For all metabolites both the fraction metabolized and elimination rates were increased. Exposure to 4hydroxyifosfamide was not changed whereas exposure to the

dechloroethylated metabolites decreased. Surprisingly, UE of 2- and 3-dechloroethylifosfamide was not decreased. This may indicate that renal clearance of 2- and 3-dechloroethylifosfamide may increase with concomitant administration of rifampicin.

In a recent preclinical study with isolated cytochrome P450 isoenzymes (cDNA supersomes) Huang et al. indicated that hydroxylation of ifosfamide is performed by CYP3A4 only, but dechloroethylation is performed by both CYP3A4 (70%) and CYP2B6 (30%).[21] The observed effects of ketoconazole in our study are in accordance with these preclinical findings. The fraction metabolized to 4-hydroxyifosfamide is inhibited, whereas the fractions metabolized to 2- and 3-dechloroethylifosfamide are uninfluenced or slightly increased. This may indicate that CYP2B6 acts as an escape-pathway for the dechloroethylation of ifosfamide. Accordingly, no qualitative differences were observed for the effect of rifampicin on the fractions metabolized to 4-hydroxyifosfamide, 2- and 3-dechloroethylifosfamide. All were increased with concomitant administration of the inducer. It should be noted that the effect of rifampicin on F^*_{4ohif} was difficult to quantitate due to the observed high correlation between F^*_{4ohif} and K_{4ohif} in the estimation procedure. Since 4-hydroxyifosfamide spontaneously decomposes no effect of rifampicin was expected on K_{4ohif}. However, a 14-fold increase was observed, which can be explained by the observed correlation. Despite the increase of the fractions metabolized to 2- and 3-dechloroethylifosfamide, their exposures were decreased due to increased elimination rates. This may indicate that metabolism of 2- and 3-dechloroethylifosfamide is induced by rifampicin as well.

From a clinical point of view, it should be noted that activation was not increased by either ketoconazole or rifampicin; corresponding fractions metabolized to 4-hydroxyifosfamide decreased or were unaffected and exposure was not influenced. Development of neurotoxicity, caused by the formation of chloroacetaldehyde, may be reflected by the fraction metabolized to 2- and 3-dechloroethylifosfamide. No decrease of the fractions was observed with either ketoconazole or rifampicin. As a result neither ketoconazole or rifampicin can be expected to produce a more favourable toxicity-efficacy profile. Co-administration of ketoconazole should even be avoided due to decreased activation.

The presented double-randomized cross-over study succeeded in establishing the effects of modulation of ifosfamide metabolism. Rifampicin increased metabolism of ifosfamide without specifically favouring the activation or deactivation route of ifosfamide. Ketoconazole decreased activation to 4-hydroxyifosfamide. Therefore, both ketoconazole and rifampicin co-administration may result in a possible decreased therapeutic benefit of ifosfamide therapy. In conclusion, our pharmacokinetic study indicated that no therapeutic benefit may be gained in humans by modulation of ifosfamide therapy with rifampicin or ketoconazole.

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Chapter 4.4

Phase I and pharmacokinetic study of the combination of topotecan and ifosfamide administered intravenously every 3 weeks -interim analysis-

Summary

Objectives were to determine the maximum tolerated dose (MTD), dose-limiting toxicities (DLT), and pharmacokinetics of topotecan administered as a 30-minute intravenous (IV) infusion over five days in combination with a 1-hour i.v. infusion of ifosfamide for three consecutive days every three weeks. Patients with advanced malignancies refractory to standard therapy were entered into the study. The starting dose of topotecan was 0.4 mg/m²/day x 5 days. Ifosfamide was administered at a fixed dose of 1.2 g/m²/day x 3 days. After identification of the MTD at the starting dose level, the topotecan administration was reduced from 5 to 3 days. Twenty-three patients received 89 treatment courses at topotecan dosages ranging from 0.4-1.2 mg/m²/day. Due to toxicities the schedule of topotecan administration was reduced from 5 to 3 days. The MTD has not yet been reached at the 3-day schedule, because DLT was observed only in one out of three patients of the highest dose-level. Haematological toxicities were dose limiting. Neutropenia was the major toxicity. Thrombocytopenia and anaemia were rare. Non-haematological toxicities were relatively mild. Pharmacokinetics of topotecan and ifosfamide and metabolites were similar to those observed after single agent administration. Modest interpatient variability (range: 14-23%) and minor interoccasion variability (range: 6-13%) in topotecan and ifosfamide clearance was observed. A sigmoidal E_{max} model could be fit to the relationship between the areas under the plasma concentration-time curve (AUC) of topotecan lactone and total topotecan and the decrease in absolute neutrophil count. Partial responses were documented in three patients with ovarian cancer. The combination of (up to) 1.2 mg/m²/day topotecan administered as a 30-minute i.v. infusion daily times 3 with 1.2 g/m²/day ifosfamide administered as a 1-hour i.v.

infusion daily times 3 every three weeks is feasible, although no MTD has been established, yet. Thus, no recommendation for phase II dosing can be made, yet. Topotecan and ifosfamide did not exhibit interactions in pharmacokinetics. Possible clinical benefit of this combination needs to be evaluated in phase II/III studies.

Introduction

Topotecan (Hycamtin®) is a semi-synthetic analogue of the alkaloid camptothecin that acts as a specific inhibitor of the nuclear enzyme topoisomerase-I.[1] Inhibition of this enzyme results in lethal DNA damage during transcription and replication. The E-ring lactone in the basic structure of topotecan is considered essential for the interaction with topoisomerase-I.[1] *In vivo*, rapid conversion of the active lactone to the inactive carboxylate form occurs. Topotecan is indicated for second-line treatment of advanced ovarian cancer or refractory small cell lung cancer and has demonstrated anti-tumour efficacy in non-small cell lung cancer, prostate cancer and colorectal cancer.[1]
Ifosfamide (Holoxan®, Ifex®) is an alkylating agent, which is indicated as third-line agent in the treatment of germ cell testicular cancer in combination with other chemotherapeutic agents. It is also used to treat cancers of the head and neck, breast, cervix, ovaries, soft tissue sarcoma, Ewing´s sarcoma, osteosarcoma, both Hodgkin´s and non-Hodgkin´s lymphomas, non-small cell lung cancer, acute lymphocytic leukaemia and neuroblastoma.[2] Ifosfamide is a prodrug, which needs to undergo activation by cytochrome P450-3A4 (CYP3A4) to 4-hydroxyifosfamide. Intracellular spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard. Ifosfamide is deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide, yielding an equimolar amount of neurotoxic chloroacetaldehyde.[3] Ifosfamide metabolism is autoinducible.[4]
In a phase I study with the combination topotecan and cyclophosphamide, an isomer of ifosfamide, synergism was suggested between alkylating agents and topoisomerase-I inhibitors.[5] It was proposed that DNA alkylation triggers DNA repair mechanisms, which may depend on topoisomerase-I. Ifosfamide was chosen as representative alkylator because it is associated with less haematological toxicity than cyclophosphamide.[2]
These data led us to evaluate a chemotherapeutic regimen of topotecan in combination with ifosfamide. Aims of this phase I trial were to determine the MTD, DLT and the safety profile of the combination of daily 30-minute infusions of topotecan on days 1-5 followed by 1.2 g/m² ifosfamide administered as a 1-hour infusion daily on days 1-3 every 3 weeks in patients with advanced malignancies. Secondary aims were the assessment of the pharmacokinetics of topotecan and ifosfamide and its main metabolites when given in combination and documentation of any anti-tumour activity of this regimen.

Patients and methods

Eligibility criteria

Eligibility criteria were histologically confirmed metastatic or locally advanced cancer, no

proven benefit of existing anti-cancer therapy, age between 18 and 75 years, World Health Organization (WHO) performance status ≤ 2, adequate bone marrow function (white blood cells (WBC) ≥ 3.5 x 10^9/L, granulocytes ≥ 1.5 x 10^9/L, platelets ≥ 100 x 10^9/L, haemoglobin ≥ 6 mmol/L), adequate hepatic function (serum bilirubin ≤ 20 µmol/L, alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT) and alkaline phosphatase (AP) ≤ 2 x the upper limit of the reference range or ≤ 5 x the upper limit of the reference range if the elevation was directly contributed to the presence of metastatic disease), adequate renal function (serum creatinine ≤ 120 µmol/L or creatinine clearance ≥ 60 ml/min), evaluable disease and a life expectancy ≥ 3 months. Exclusion criteria were breast feeding, pregnancy or inadequate contraceptive protection, drug, medication or alcohol abuse, treatment with an investigational drug ≤ 1 month prior to the study-entry, surgery, radiotherapy (except for analgesic indications) or chemotherapy (mitomycin C or nitrosoureas ≤ 1.5) ≤ 1 month prior to the study-entry, other diseases altering procedures of the trial, history of seizures or central nervous system disorders, severe intercurrent infection, serious uncontrolled concurrent medical or psychiatric disease, known hypersensitivity to ifosfamide or topotecan, prior treatment with ifosfamide or topotecan. The study protocol was approved by the Ethics Review Boards of the Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute and University Medical Center Utrecht. The trial was conducted in these two centres according to Good Clinical Practice and the current edition of the Declaration of Helsinki. Written informed consent was obtained from all patients entering the study.

Toxicity and response evaluation

Pre-treatment evaluation included a complete medical history and complete physical examination. Before each course, blood chemistry and haematological profiles were checked and urinalysis was performed. Complete blood cell counts and blood chemistry were repeated weekly. Electrocardiograms and tumour measurements (by physical examination, chest x-ray, other radiological investigations, or ultrasound) were performed every other cycle. All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC).[6] DLTs were defined as any of the following events occurring during the first treatment cycle and attributable to either ifosfamide or topotecan: (1) grade IV neutropenia lasting ≥ 7 days or of any duration associated with severe systemic infection requiring parental treatment, (2) febrile neutropenia, (3) grade III or IV thrombocytopenia with or without haemorrhagic complications, or (4) grade III or IV non-haematological toxicity excluding nausea/vomiting responsive to treatment, anorexia and alopecia. The MTD was defined as the dose at which ≥ two out of three or ≥ two out of six patients experienced DLT. The next lower dose level below the MTD was the recommended dose for phase II studies. Responses were determined according to the WHO criteria.[7]

Dose escalation

Doses of topotecan were escalated during the study. Starting dose was 0.4 mg/m²/day topotecan for 5 days by means of a 30-minute infusion. Escalation was planned with 0.2

mg/m²/day increments per dose level. Ifosfamide was administered at a fixed dose of 1.2 g/m²/day for 3 days by means of a 1-hour infusion. Ifosfamide was administered directly after the topotecan infusion. Upon identification of the MTD, the protocol was amended to reduce topotecan dosing from 5 to 3 days. Treatment cycles were repeated every 21 days, provided patients had sufficiently recovered from any drug-related toxicity associated with the previous course (non-haematological toxicity ≥ 1, alopecia excluded, haemoglobin ≥ 6 mmol/L and return of blood cell counts to ≥ 1.5 x 10^9/L neutrophils and ≥ 100 x 10^9/L platelets). Dose modifications of both ifosfamide and topotecan were made if poor subjective tolerance to treatment occurred. Neutrophils < 0.5 x 10^9/L associated with temperature ≥ 38.5 °C or lasting longer ≥ 7 days, neutrophils < 1.0 x 10^9/L lasting longer ≥ 21 days, platelets < 50 x 10^9/L or any non-haematological toxicity requiring a delay in treatment resulted in dose reduction of 25% and platelets < 25 x 10^9/L resulted in dose reduction of 50%.

Drug administration

Topotecan (Hycamtin®) was supplied by SmithKline Beecham Farma (Rijswijk, The Netherlands) in vials containing topotecan HCl, equivalent to 5 mg of free base. Inactive ingredients were 60 mg mannitol and 25 mg tartaric acid. The content of each vial was reconstituted with 2 ml sterile water for injection. The appropriate dosage of the drug was diluted in 50 ml of 0.9% NaCl solution. Topotecan was administered through a peripheral venous access device using a syringe pump. Ifosfamide (Holoxan®) was supplied by Asta Medica BV (Diemen, The Netherlands) in vials containing 0.5, 1 or 2 g without excipients. Ifosfamide was diluted in 0.9 % NaCl to approximately 1 l and administered through a peripheral venous access device using an ambulatory infusion pump.

Pharmacokinetic sampling

Pharmacokinetic studies were performed for topotecan (total and lactone form), and ifosfamide and metabolites (2- and 3-dechloroethylifosfamide, 4-hydroxyifosfamide and ifosforamide mustard) during the first two treatment courses. Intravenous samples were drawn from an indwelling intravenous cannula placed in the arm contralateral to the arm receiving chemotherapy prior to the topotecan infusion and at the following time-points after start of the topotecan infusion: 15, 30 (prior to the end of the topotecan infusion), 60 and 90 min (prior to the end of the ifosfamide infusion), and 2, 3, 4.5, 7.5, 11 and 24 hours (prior to the start of the following topotecan infusion). Pharmacokinetic sampling was executed according to this schedule on days 1 and 3 of cycle 1 and day 1 of cycle 2. Blood samples were immediately placed on ice water. The plasma was immediately separated by centrifugation at 1,000 g for 5 min at 4°C. For the analysis of topotecan lactone plasma protein precipitation was performed by adding 1 ml plasma to 2 ml cold methanol (-20°C). The sample was mixed on a whirl mixer for 10 sec. and centrifuged for 3 min at 1,000 g at 4°C. The clear supernatant was transferred to a polypropylene tube and immediately stored at -70°C pending analysis. Furthermore, plasma was aliquoted in three 1-ml volumes. Two aliquots were used for 4-hydroxyifosfamide analysis after stabilization with semicarbazide and

one aliquot was used for ifosforamide mustard analysis after stabilization with semicarbazide and sodium chloride. The remaining plasma was aliquoted to two 0.5-ml volumes for analysis of ifosfamide, 2- and 3-dechloroethylifosfamide, and total topotecan. Urine was collected during the first course for 96 hours after the start of the ifosfamide infusion and an aliquot was analysed for ifosfamide, 2- and 3-dechloroethylifosfamide. Both plasma and urine samples were immediately stored at -70°C, pending analysis. Total topotecan (lactone plus carboxylate form) and the lactone form were determined separately using a high-performance liquid chromatographic (HPLC) system with fluorometric detection.[8] Gas chromatography with selective nitrogen-phosphorous detection was used for the determination of ifosfamide, 2- and 3-dechloroethylifosfamide in urine and plasma.[9] Two separate HPLC methods were used for the determination of 4-hydroxyifosfamide and ifosforamide mustard concentrations.[10,11]

Pharmacokinetic evaluation

The pharmacokinetics of lactone and total topotecan were described with a two-compartment model, yielding clearance (CL, $L \cdot h^{-1}$), volume of distribution (V, L), volume of distribution at steady-state (Vss, L) and intercompartmental clearance (Q, $L \cdot h^{-1}$). The pharmacokinetics of ifosfamide and its main metabolites were described using an integrated non-linear model with autoinduction.[4,12,13] This model described the pharmacokinetics of ifosfamide with an initial clearance of ifosfamide (CL_{init}, $L \cdot h^{-1}$) and a volume of distribution (V_{ifo}, L), and autoinduction with a first-order rate constant for enzyme degradation/inactivation ($K_{enz,out}$, h^{-1}) and an ifosfamide concentration at 50% of the maximum inhibition of enzyme degradation (IC_{50}, µM). The induction half-life of the enzyme ($t_{½,enz}$, h) was calculated by the ratio of ln(2) and $K_{enz,out}$. The pharmacokinetics of the metabolites were described with a ratio of the fraction of ifosfamide metabolized and the volume of distribution of that metabolite ($F_m/V_m=F^*$, L^{-1}), an elimination rate constant (K, h^{-1}) and a rate constant (F^{**}) from 4-hydroxyifosfamide to ifosforamide mustard.[13] The exposure to a compound was described by the area under the plasma concentration-time curve (AUC). All pharmacokinetic models were fitted to the data from the individuals using the population pharmacokinetic programme NONMEM (Non-linear Mixed Effects Modelling, Version V).[14] The residual or intraindividual variability of the pharmacokinetics were described separately with a combined proportional and additive term. The interindividual variability of each pharmacokinetic parameter was estimated using a proportional error model.

Pharmacokinetic-pharmacodynamic relationships

Relationships between the AUC of lactone and total topotecan and myelosuppression were explored using scatter plots of the AUC versus the percentage decrease in absolute neutrophil count (ANC). The percentage decrease in blood cells was defined as:

$$\%\,\text{decrease} = \frac{\text{baseline count} - \text{nadir count}}{\text{baseline count}} \times 100\%$$

The data were fit to a sigmoidal maximum effect (E_{max}) model, as described by the modified Hill equation[15]:

$$\%\ \text{decrease} = \frac{\left(E_{max} \times DE^{\gamma}\right)}{\left(DE_{50}^{\gamma} + DE^{\gamma}\right)}$$

where E_{max} denotes the maximal effect that can be elicited, DE is a measure of drug exposure (i.e. AUC), DE_{50} represents the drug exposure associated with 50% of E_{max}, and γ is the Hill-coefficient describing the sigmoidicity of the curve. Modelling was performed using S-PLUS (Professional release 1, Mathsoft Inc.)

Results

Patients

At the cut-off date for the interim analysis (11 August, 2000) 23 patients have received 89 courses at six dose levels. Patient characteristics are listed in table 1. The medium number of courses administered per patient was 4 (range 1 to 9). In three patients only one treatment course was administered because of rapid progressive disease. Two of these patients developed an ileus, one with septic shock and one with lethal intestinal bleeding, which toxicity was considered disease-related. Two courses were delayed in one patient at dose level 5 (1.0 mg/m²/day x3) due to the occurrence of an ileus.

Dose escalation

All patients were assessable for toxicity during the first treatment course. Doses of topotecan were escalated according to the scheme depicted in table 2. Myelosuppression was the dose-limiting toxicity for the combination of topotecan and ifosfamide in this schedule (table 3). At dose level 2 (topotecan 0.6 mg/m²/day x 5) one patient developed dose-limiting grade III thrombocytopenia, grade IV neutropenia and grade IV anaemia (due to intestinal bleeding) resulting in toxic death.

Table 1. *Patient characteristics*

Characteristic	Number	%
Number of patients	23	
Male/female	3/20	
Age, years		
Median	58	
Range	37-75	
Performance status		
0	7	30
1	13	57
2	3	13
Primary tumour site		
Colon	1	4
Cervix	1	4
Melanoma	1	4

Ovarian	15	65
Gastric	1	4
Renal	1	4
NSCLC	2	9
NSCLC+colon	1	4
Prior palliative chemotherapy	21	91
Number of previous regimens		
Median	1	
Range	1-3	
Prior radiotherapy	3	13

NSCLC=non-small cell lung cancer.

Table 2. *Topotecan dose escalation*

Dose level	Number of patients	Topotecan dose	Total topotecan dose
1	5	0.4 mg/m²/day x 5days	2.0 mg/m²
2	3 (DLT in 1 of 3)	0.6 mg/m²/day x 5days	3.0 mg/m²
3	6	0.6 mg/m²/day x 3days	1.8 mg/m²
4	3	0.8 mg/m²/day x 3days	2.4 mg/m²
5	3	1.0 mg/m²/day x 3days	3.0 mg/m²
6	3 (DLT in 1 of 3)	1.2 mg/m²/day x 3days	3.6 mg/m²

Accrual at dose level 2 was stopped and two additional patients were treated at dose level 1; no DLT was observed in these patients. To investigate further dose-intensification topotecan dosing was reduced from 5 to 3 days, yielding a total dose at level 3 similar to level 1. At dose level 3 only 5 courses of chemotherapy could be evaluated in the first three patients, because of the development of progressive disease. Therefore, it was decided to include 3 additional patients at level 3.

Table 3. *Haematological toxicities in 70 of 89 courses.*

Dose level	Topotecan dose	Number of patients	Number of courses	Anaemia*		Neutropenia*		Thrombocytopenia*	
				Grade III	Grade IV	Grade III	Grade IV	Grade III	Grade IV
1	0.4 mg/m²x5d	5	20	0/1	0/0	1/1	0/2	0/0	0/0
2	0.6 mg/m²x5d	3	10	0/0	1/1	0/0	2/4	1/1	0/0
3	0.6 mg/m²x3d	6	17	0/0	0/0	0/6	2/4	0/0	0/0
4	0.8 mg/m²x3d	3	8(21)**	0/0	0/0	0/1	1/3	0/0	0/0
5	1.0 mg/m²x3d	3	7(10)**	0/1	0/0	0/1	3/5	0/0	0/0
6	1.2 mg/m²x3d	3	8(10)**	0/0	0/0	0/0	2/6	1/1	0/0

*number of patients developing toxicity in the 1st course/number of courses causing toxicity, **number of courses evaluated of total.

Table 4. *Nadir blood counts in 70 of 89 courses.*

Dose level	Topotecan dose	Number of patients	Number of courses	ANC (x10^9/L)		Haemoglobin (mmol/L)		Platelets (x10^9/L)	
				1st course	All courses	1st course	All courses	1st course	All courses
				Median (range)	Median (range)	Median (range)	Median (range)	Median (range)	Median (range)
1	0.4 mg/m² x 5d	5	20	1.9 (0.7-3.5)	2.0 (0.4-3.5)	6.4 (4.4-7.1)	6.5 (1.2-5.9)	230 (99-403)	165 (99-445)
2	0.6 mg/m² x 5d	3	10	0.4 (0.2-1.3)	1.0 (0.2-2.4)	5.6 (3.4-5.7)	5.7 (3.4-6.6)	79 (25-231)	212 (25-282)
3	0.6 mg/m² x 3d	6	17	1.4 (0.1-3.7)	0.7 (0.04-4.0)	6.2 (5.9-7.0)	5.9 (5.0-7.3)	190 (125-283)	175 (78-283)
4	0.8 mg/m² x 3d	3	8(21)**	2.1 (0.3-3.5)	1.0 (0.3-3.5)	6.1 (5.7-7.5)	6.7 (5.7-7.5)	176 (129-230)	235 (129-261)
5	1.0 mg/m² x 3d	3	7(10)**	0.2 (0.1-0.3)	0.3 (0.1-0.7)	6.2 (5.5-6.8)	5.6 (4.0-6.8)	267 (193-269)	269 (130-399)
6	1.2 mg/m² x 3d	3	8(10)**	0.1 (0.02-2.1)	0.2 (0.02-2.1)	5.6 (5.5-6.1)	5.6 (5.3-6.1)	71 (44-117)	107 (44-419)

**number of courses evaluated of total.

Dose levels 4 and 5 were uneventful. At level 6 dose-limiting grade IV neutropenia accompanied by neutropenic fever for more than 7 days was observed in one out of three patients. Therefore another 3 patients will be entered at this level.

Haematological toxicity

Myelosuppression was the principal toxicity. Table 3 lists the frequency of the grade III and IV haematological toxicities during treatment course 1 and during all courses. A total of 70 of 89 courses were evaluated so far involving all 23 patients. Overall, grade III thrombocytopenia (3%) and grade III (3%) and IV (1%) anaemia were rare. Grade IV anaemia was considered not related to the study drugs. Neutropenia grade III and IV occurred in 9 (13%) and 24 (34%) of all courses, respectively. Nine (10%) of all evaluable treatment courses were delayed for one week. Three patients had unresolved neutropenia at day 21, one patient had two episodes of ileus, one patient had two episodes of severe ascitis, and two courses were postponed upon patients' request. Dose reductions due to neutropenia were performed in 12 (17%) of 70 treatment courses involving five patients. At dose level 2 (0.6 mg/m²/day x5) one patient received two courses at 75% of the intended dose and another patient received four courses with topotecan for 3 days instead of 5 days. At dose level 3 one patient received one course at 75% of the intended topotecan dose. At dose level 6 one patient developed grade IV neutropenia with fever, which was resolved before start of the second course. However, the fever reoccurred during the second day of the second course. This prompted an early infusion discontinuation after the first day of the second course. At the same dose level another patient experienced DLT (grade III thrombocytopenia, grade IV neutropenia with neutropenic fever). Consequently, the dose of topotecan and ifosfamide was reduced by 25% in all subsequent courses in this patient.
There was no indication of cumulative myelosuppression on repeated dosing, evidenced by similar or less prominent haemoglobin, neutrophil and platelet nadirs in later courses compared with the first course (table 4). The neutrophil nadir typically occurred between days 7 and 14. Absolute neutrophil counts at dose level 2 (dose intensity 3.0 mg/m²) were comparable to dose level 5 and 6 (dose intensities 3.0 and 3.6 mg/m², respectively). A similar pattern was observed with the haemoglobin and platelets levels of dose levels 2 and 6. Most patients in the study received infusions of packed cells due to anaemia.

Non-haematological toxicity

Non-haematological toxicities were relatively mild and not dose-related. Thus far, a total of 20 patients with 61 courses were evaluated for non-haematological toxicities. Nausea and/or vomiting grade III was observed in four (7%) courses involving three patients. Transient nausea and/or vomiting grade I or II was observed in 67% of the assessable courses and responded well to standard anti-emetics. Other treatment related gastrointestinal side effects of grade I or II included abdominal cramps (3%), constipation (48%) and anorexia (5%). In addition, one patient suffered from grade III diarrhoea. Fatigue grade III was observed in two (3%) courses in one patient and grade I or II in 21 (34%) courses. Fatigue grade II in two patients resulted in delay of treatment at the patients' request. Fever grade III was observed in

one patient and grade II in 7 (11%) courses involving five patients. Asthenia grade III was observed in 3 (5%) courses involving three patients and grade I and II in another 12 (20%) courses. Alopecia grade I and II was observed in 16 (70%) patients without influence of dose. Peripheral neuropathy grade I was observed in 9 (15%) courses involving four patients, of which one patient (6 courses) had pre-existing neuropathy due to cisplatin pre-treatment. In one patient an episode of neuropathy grade II was suspected to be treatment-related, but in another patient an episode of grade III was not considered to be related, because of confirmed CNS metastases. One patient complained of mild dizziness. All toxicities including neuropathy were reversible and rapidly disappeared after therapy was stopped.
Blood chemistry prior to treatment did not exceed reference values. Median creatinine clearance prior to treatment was 85 ml/min and ranged between 48 and 136 ml/min; this was unaltered prior to the second course. In one patient increased levels of AP, ASAT and ALAT (946, 103 and 132 U/L, respectively) were observed after the first course. Later, this patient was found to have an obstructed biliary tract due to tumour growth. Between course 1 and 2 a total of three and two patients had calcium and magnesium levels below the reference values of 2.2 and 0.7 µmol/L, respectively.

Pharmacokinetics

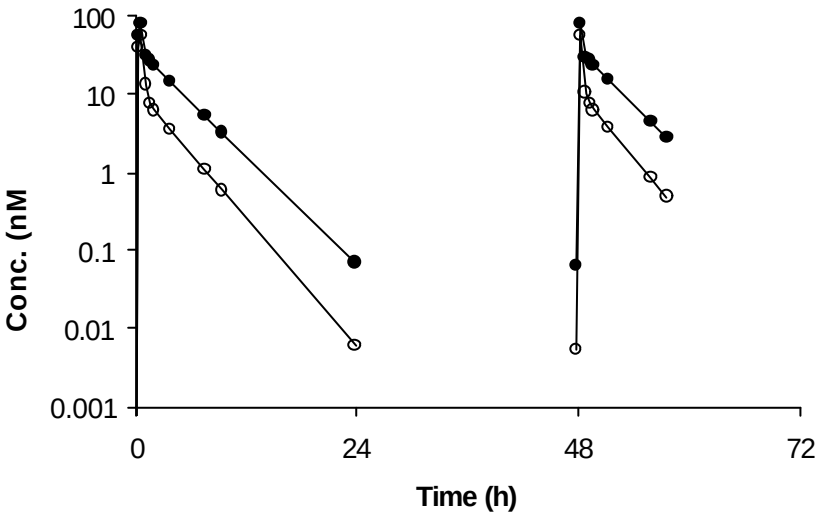

Figure 1. *A typical plasma concentration-time profile of total topotecan (solid markers) and topotecan lactone (open markers) of a patient receiving a 30-minute administration of 1.2 mg/m²/day topotecan for three consecutive days.*

Complete pharmacokinetic data sets were obtained in the first course of all 23 patients and in the second course of 17 patients. Typical plasma profiles of topotecan lactone and total topotecan of a patient receiving 1.2 mg/m²/day topotecan are displayed in figure 1.
The lactone form was the predominant form during the infusion, but approximately 30 minutes after the end of the infusion the open-ring (carboxylate) form concentrations exceeded lactone levels. The mean (±sem) lactone-to-total topotecan ratio at the end of the infusion was 0.74±0.02 (n=23). After the end of the infusion, lactone curves exhibited a bi-exponential

decay. The population pharmacokinetic parameters of topotecan lactone and total topotecan are listed in table 5.

Table 5. *Pharmacokinetics of topotecan lactone and total topotecan (lactone and carboxylate form) with their relative standard error of the mean (sem), interindividual variability (IIV), interoccasion variability (IOV) and residual variability (RV).*

	topotecan lactone				total topotecan			
	Mean (±sem)	IIV	IOV	RV	Mean (±sem)	IIV	IOV	RV
CL (L· h^{-1})	65.7±3.0	14%	9%		24.4±1.4	23%	13%	
V (L)	27.9±1.1	22%	-		16.5±2.5	40%	-	
Vss (L)	123±5	13%	-		77.0±3.0	15%	-	
Q (L· h^{-1})	65.3±2.6	-	-		112±13	60%	-	
PE				14.%	PE			12.8%
AE (µM)				0.143	AE (µM)			0.406

CL=clearance, V=volume of distribution of ifosfamide, Vss=volume of distribution at steady-state, Q=intercompartmental clearance, PE=proportional error, AE=additive error.

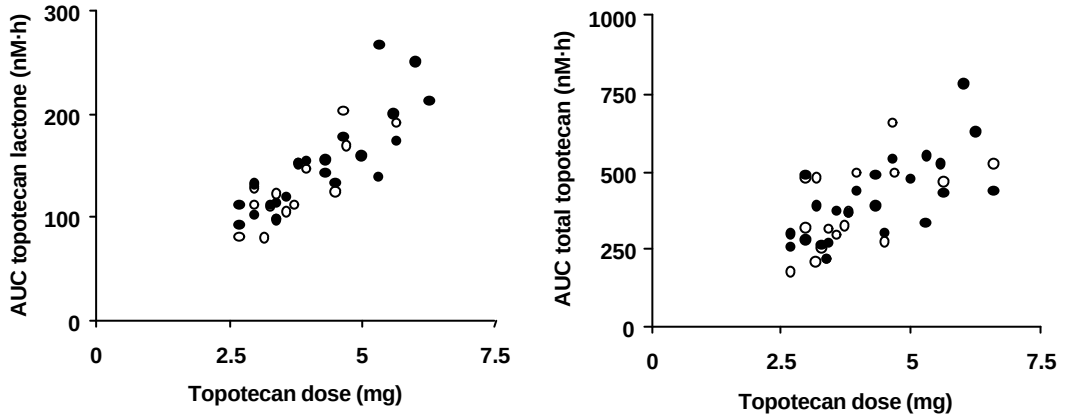

Figure 2. *The relationship between the total topotecan dose (mg) per course and the area under the plasma concentration-time curve (AUC) of total topotecan (upper graph) and topotecan lactone (lower graph) in course 1 (solid marker) and 2 (open marker).*

Interindividual variability was only modest (14-60%). For the intercompartmental clearance of topotecan lactone inclusion of the interindividual variability in the pharmacokinetic model did not improve the fit. This should not be interpreted as an absence of interindividual variability in this parameter, but only that the concentration-time data did not contain sufficient information for the model to estimate it. Interoccasion variability in clearance of topotecan lactone and total topotecan was small with 9 and 13%, respectively. No interoccasion variability could be estimated for the other parameters. The relationship between the AUC of lactone and total topotecan with the absolute topotecan dose is represented in figure 2.

Plasma concentration-time profiles of ifosfamide and its metabolites of a typical patient receiving 1.2 g/m²/day ifosfamide are presented in figure 3. A decrease in elimination half-life of ifosfamide was observed when comparing the mono-exponential decays on day 1 and 3,

due to the autoinduction of the ifosfamide metabolism. This effect was reset at the start of the next course. The population pharmacokinetic parameters of ifosfamide and its metabolites are listed in table 6. The concentration-time data did not allow estimation of IC_{50} and it was therefore fixed at 0.001 µM, which was far below the lower limit of quantification. Fixation of IC_{50} resulted in an adequate description of the development of autoinduction of the ifosfamide metabolism. Interindividual variability in ifosfamide pharmacokinetics was modest (14-22%) and somewhat larger for the metabolites (29-76%). Interoccasion variability of the initial clearance of ifosfamide was small (6%) and could not be estimated for the other parameters. Mean (±sem) urinary excretion of ifosfamide was 34.7±2.7% of the administered dose with 17.8±2.2% as unchanged ifosfamide, 5.6±0.5% as 2-dechloroethylifosfamide and 11.2±0.9% as 3-dechloroethylifosfamide.

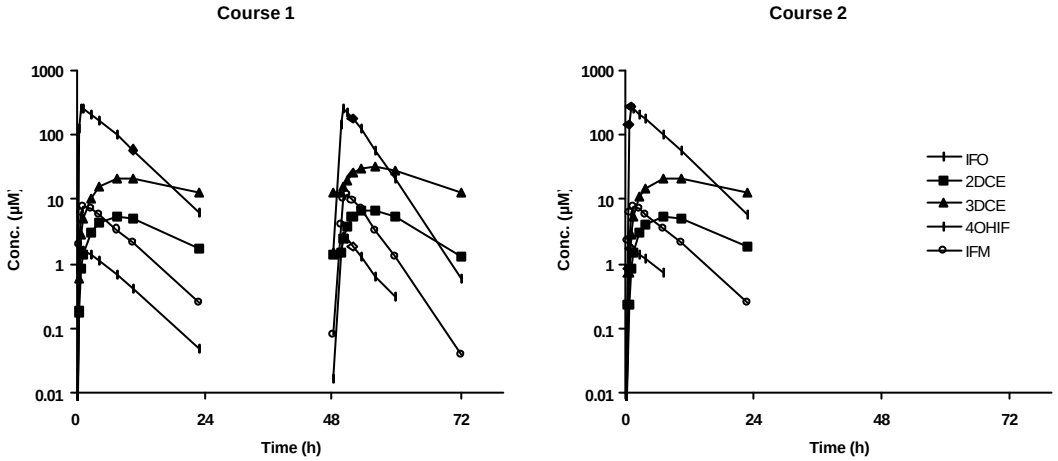

Figure 3. *Typical plasma concentration-time profiles of ifosfamide (IFO) 2-dechloroethylifosfamide (2DCE), 3-dechloroethylifosfamide (3DCE), 4-hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) of a patient receiving a 1-hour infusion of 1.2 g/m²/day ifosfamide for three consecutive days with a 3-week interval.*

Pharmacodynamics

The correlations between the AUC for the lactone and total topotecan and the percentage decrease in neutrophils could adequately be described by sigmoidal E_{max} models (figure 4). Estimations for E_{max}, AUC_{50} and the Hill-coefficient of the four models are depicted in table 7.

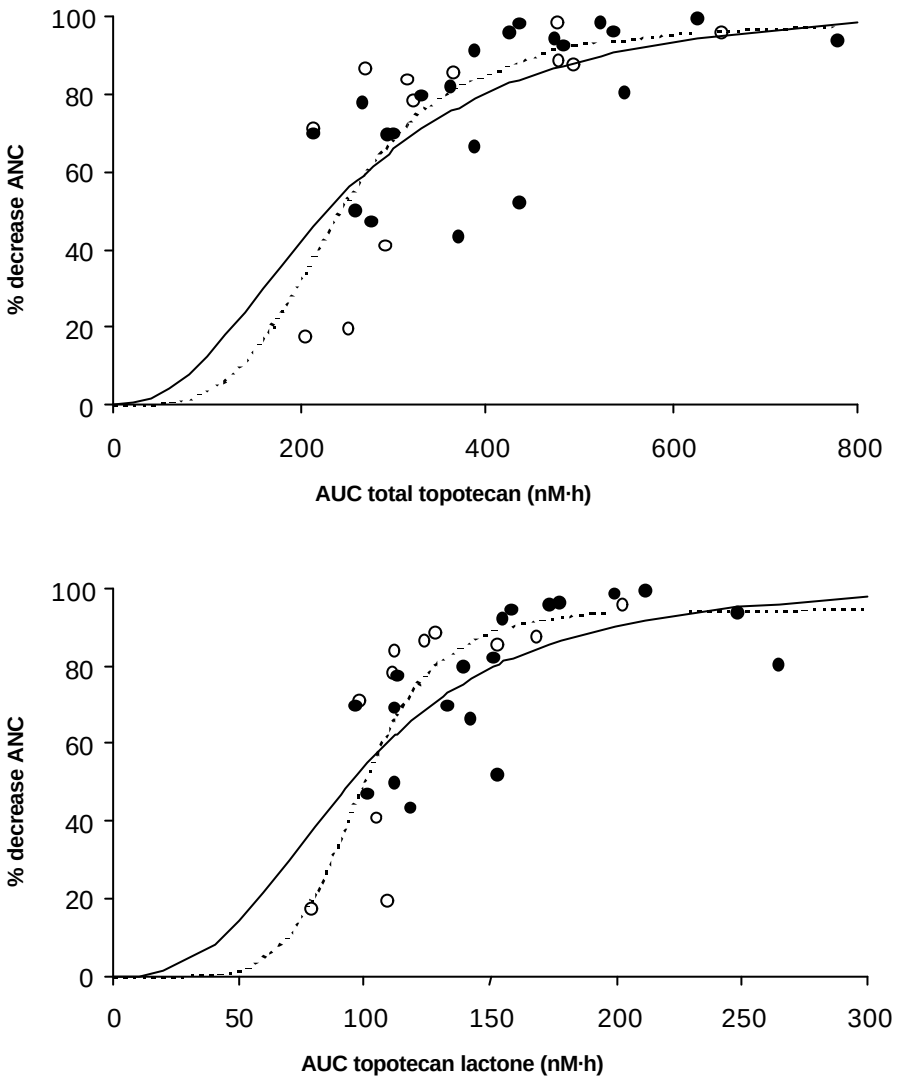

Figure 4. *The percentage decrease in absolute neutrophil count (ANC) versus the area under plasma concentration-time curve (AUC) of total (upper graph) and lactone (lower graph) topotecan during course 1 (solid markers) and course 2 (open markers). The lines indicate the best fit of the data to the sigmoidal maximum effect pharmacodynamic model in course 1 (solid line) and course 2 (dashed line).*

Responses

Eighteen patients were assessable for therapeutic activity. Eleven patients demonstrated stable disease and four patients progressive disease during treatment. Partial responses were documented in three patients heavily pre-treated for ovarian cancer.

Table 6. *Pharmacokinetics of ifosfamide (IF), 2dechloroethylifosfamide (2DCE), 3dechloroethylifosfamide (3DCE), 4hydroxyifosfamide (4OHIF) and ifosforamide mustard (IFM) with their relative standard error of the mean (sem), interindividual variability (IIV), interoccasion variability (IOV) and residual variability (RV).*

	Ifosfamide					Deactivated metabolites				Activated metabolites		
	Mean (±sem)	IIV	IOV	RV		Mean (±sem)	IIV	RV		Mean ± RE	IIV	RV
CL_{init} (L/h)	3.52±0.18	22%	6%		F^{*}_{2DCE} (L^{-1})	0.00406±0.00077	70%		F^{*}_{4OHIF} (L^{-1})	0.147±0.013	43%	
V_{IFO} (L)	32.3±1.0	14%	-		K_{2DCE} (h^{-1})	0.118±0.021	27%		K_{4OHIF} (h^{-1})	73.8	-	
$K_{enz,out}$ (h^{-1})	0.0137±0.0012	20%	-		AUC_{2DCE} (µM· h)	0.861±0.132			AUC_{4OHIF} (µM· h)	0.0482±0.0044		
$t_{½,enz}$ (h)	50.6				PE			0%	PE			23.0%
AUC_{IFO} (µM· h)	4.67±0.27				AE (µM)			5.10	AE (µM)			0.274
PE				14.3%	F^{*}_{3DCE} (L^{-1})	0.00633±0.00044	29%		F^{**}_{IFM} (h^{-1})	0.56±0.36	-	
AE (µM)				4.51	K_{3DCE} (h^{-1})	0.0752±0.0038	-		K_{IFM} (h^{-1})	4.0±1.5	76%	
					AUC_{3DCE} (µM· h)	2.04±0.13			AUC_{IFM} (µM· h)	0.399±0.089		
					PE			17.3%	PE			10.7%
					AE (µM)			3.22	AE (µM)			4.50

CL_{init}=initial ifosfamide clearance, V_{IFO}=volume of distribution of ifosfamide, $K_{enz,out}$=first-order rate constant for enzyme degradation/inactivation, $t_{½,enz}$=induction half-life of the enzyme, AUC=area under the plasma concentration-time curve during the first course, PE=proportional error, AE=additive error, F^{*}=ratio of fraction metabolized and volume of distribution of metabolite, K=first-order rate constant for metabolite elimination, F^{**}=formation rate constant.

Table 7. *The estimated parameters of the relationship between the area under the plasma concentration-time curve (AUC) of lactone and total topotecan and the percentage decrease in absolute neutrophil count (ANC) in course 1 and 2 of 23 patients.*

	topotecan lactone		total topotecan	
	Mean (±sem)		Mean (±sem)	
	Course 1	Course 2	Course 1	Course 2
E_{max} (%)	101±20	95±16	105±25	97±20
AUC_{50} (µM)	95±12	98±9	239±48	243±36
γ	2.8±2.0	6.3±4.1	2.3±1.4	3.8±2.7

E_{max}=maximum effect on ANC, AUC_{50}=topotecan AUC associated with 50% of E_{max}, γ=Hill-coefficient

Discussion

There are few anticancer agents that have significant therapeutic impact when used alone, particularly in the treatment of solid tumours. The single agent and combination therapy activities of topotecan and ifosfamide have been extensively studied.[1,2] Murren et al. proposed a synergistic cytotoxicity between topoisomerase-I inhibitors and alkylating agents.[5] Bioactivation of ifosfamide leads to the alkylating compound ifosforamide mustard, which possesses a high affinity for macromolecules such as DNA. DNA alkylation leads to cross-link formation finally resulting in cytotoxicity and cell death through apoptosis. DNA alkylation initiates DNA repair mechanisms. Topoisomerase-I may be involved in this machinery through unwinding of the DNA. However, at present its direct relationship to the cytotoxic events of ifosfamide is unclear. The suggested synergism encouraged us to evaluate the feasibility and safety profile of the combination topotecan with ifosfamide in cancer patients.

Haematological toxicity, especially neutropenia was the dose-limiting toxicity for this combination. Topotecan could not be safely administered in the approved single agent (1.5 mg/m²) daily times five schedule. Severe neutropenia and thrombocytopenia occurred at a dose of 0.6 mg/m^2/day for 5 days. To investigate further dose intensification, topotecan dosing was reduced from 5 to 3 days. With the adjusted schedule, topotecan doses could be escalated to 1.2 mg/m^2/day. The currently tested daily dose of 1.2 mg/m²/day topotecan in combination with 1.2 g/m²/day ifosfamide for three days resulted in DLT in one of three patients. Therefore, another three patients need to be included.

The neutropenia was correlated with the dose-intensity of topotecan (table 3), which was also observed in previous studies.[16] Neutropenia is the most frequently observed toxicity associated with single agent topotecan. In phase II trials of single agent topotecan in adult patients with solid tumours receiving 30-minute infusions of 1.5-2.0 mg/m²/day for 5 days every 3 or 4 weeks, grade III to IV neutropenia was observed in 71 to 75% of all courses.[17,18] The haematological toxicity was generally of short duration, non-cumulative and manageable. Ifosfamide's principal toxicity is also haematological, albeit less severe than its isomer cyclophosphamide. A WBC count < 3 x 10^9/L is expected in approximately 50% of patients and platelet counts < 100 x 10^9/L in 20% of patients treated with single agent ifosfamide at a dose of 1.2 g/m²/day for 5 days.[19] Their over-lapping toxicity profiles prevented escalation of topotecan in combination with ifosfamide, to a dose near the single agent MTD of 1.5 mg/m^2/day for 5 consecutive days.

Bone marrow colony-stimulating factors (G-CSF) could potentially improve the tolerance to the combination regimen. However, preliminary results presented by Bienvenu et al. suggest the impact of G-CSF to be limited.[20] They recommended a dose of 1.0 mg/m²/day topotecan in combination with 1.5 g/m²/day ifosfamide both daily for three consecutive days with G-CSF support from days 5 to 12.

Two episodes of grade III anaemia were observed in all courses, which were considered related to the study drugs. This is in accordance with the observation that significant anaemia occurred in 2-14% of all courses of phase II studies in patients receiving single agent topotecan at 1.5-2.0 mg/m²/day for 5 days.[17,18]

Only two episodes of grade III thrombocytopenia were observed in our study.

Thrombocytopenia associated with single agent topotecan is rare and mainly reported after prolonged infusion schedules.[21] Severe thrombocytopenia was observed in 2-10% of all courses in the above mentioned phase II studies of the daily times 5 schedule.[17,18]
All non-haematological toxicities were relatively mild, not dose-related and transient with nausea and/or vomiting, fatigue, constipation and alopecia being the most observed. These have been described before in phase II studies of single agent topotecan with similar incidence rates.[18] Peripheral neuropathy or paresthesia occurred rarely with topotecan treatment and a causal relationship is uncertain. The relatively high incidence of neuropathy in our study may be related to prior treatment with paclitaxel in 3 and cisplatin in 1 of 5 patients.[22] None of the specific ifosfamide toxicities have been observed in this study, such as renal tubular abnormalities, haemorrhagic cystitis and encephalopathy.[2]
Population pharmacokinetic parameters of topotecan were similar to previously reported parameter estimates obtained using a population approach and a standard two stage analysis after single agent administration of 0.5-2.0 mg/m² topotecan in the daily times 5 schedule.[23,16,24] Metabolic interactions of topotecan with concomitantly administered ifosfamide were not anticipated, because topotecan metabolism to N-desmethyl-topotecan is only 0.4-3% of the dose.[21]
Nephrotoxicity is frequently encountered with ifosfamide treatment. Since approximately 40% of topotecan is eliminated by the kidneys, ifosfamide-induced renal tubular damage could potentially alter the renal clearance of topotecan. In the current study no episodes of renal toxicity were observed. Creatinine clearance, an indicator of renal function, did not exceed reference values. In agreement with these clinical findings, plasma profiles of topotecan on days 2 and 3 were similar to those on day 1 indicating that ifosfamide co-administration at the applied dose regimen most likely does not interfere with topotecan clearance. However, an assessment of the urinary excretion of topotecan will be made to exclude decreased renal clearance of topotecan by ifosfamide.
Population pharmacokinetic parameters of ifosfamide and metabolites were similar to those reported in a previous pharmacokinetic study using an identical model.[13] In another study high doses of ifosfamide (9-12 g/m^2) were administered as single agent by a 72-hour continuous infusion.[4] A four-fold higher value for $t_{½,enz}$ was obtained in the current study. Furthermore, no IC_{50} could be estimated. This may be explained by the fact that estimation of autoinduction in a 1-hour infusion is inherently less feasible than in a 72-hour continuous infusion of ifosfamide. In conclusion, although the analysis is not fully complete topotecan and ifosfamide do not appear to interact pharmacokinetically in the studied combination regimen.
The estimates for the parameters used to describe the sigmoidal E_{max} relationship of the pharmacokinetics and the myelosuppression were similar to previously reported values.[1] Warmerdam et al. reported an AUC_{50} and Hill-coefficient for total topotecan of 173 nM· h and 1.8, respectively, after single agent topotecan (0.5-1.5 mg/m²/day x5).[16,25] No differences were observed in the estimates for the first and second course in the current study. This is in accordance with the observation that neutropenia is not cumulative. So far, dose escalation of topotecan only reached approximately 50% of the MTD of single agent treatment with topotecan. This indicates a considerable additive myelosuppressive effect of ifosfamide. However, no clear relationship between the exposure to the activated metabolites of ifosfamide (4-hydroxyifosfamide and ifosforamide mustard) and the myelosuppression was

observed. Preliminary investigations indicated that combined modelling of topotecan and ifosfamide do not result in an increased goodness-of-fit of the relationship with myelosuppression.

It is concluded that the combination of topotecan and ifosfamide is feasible, although for the 3-day schedule no MTD has yet been established. Both drugs demonstrated single agent pharmacokinetics without apparent mutual interactions. Possible clinical benefit of this combination needs to be evaluated in future trials.

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Summary and conclusions

Introduction

Ifosfamide is an alkylating agent, which is used in the treatment of various types of malignant diseases in adults and childeren. Its use, however, can be accompanied by severe haematological, neuro- and nephrotoxicities. Since its development in the middle of the 1960's, most of its extensive metabolism has been elucidated. Ifosfamide is a prodrug, which needs to undergo activation by cytochrome P450-3A4 (CYP3A4) to 4-hydroxyifosfamide. Intracellular spontaneous decomposition of 4-hydroxyifosfamide yields the ultimate alkylating metabolite ifosforamide mustard. Ifosforamide mustard binds to DNA causing cross-links followed by apoptosis. Ifosfamide is deactivated to the non-cytotoxic metabolites 2- and 3-dechloroethylifosfamide, yielding an equimolar amount of neurotoxic chloroacetaldehyde. Ifosfamide metabolism is autoinducible. In chapter 1 a review of the literature is given addressing key issues in the clinical pharmacology of ifosfamide and its metabolites.
Understanding the relationship between plasma concentrations (pharmacokinetics) and effect (pharmacodynamics) is important for the rational development and the safe and effective use of every therapeutic agent. Since the efficacy and specific toxicities of the treatment with ifosfamide may be linked to its extensive metabolism, pharmacokinetic assessment of ifosfamide and its metabolites is of special interest in helping to explain the unpredictable chances of success and failure in ifosfamide treatment. To characterize the pharmacokinetic-pharmacodynamic relationships and their variability adequately, studies in a representative population using a relatively large number of patients, are needed. However, for practical and ethical reasons extensive pharmacokinetic and pharmacodynamic studies in a large number of often critically ill patients are not possible. Therefore, there was a need for an approach with the help of which description of the relevant pharmacokinetic and pharmacodynamic relationships and of their variability is possible on the basis of sparse data (few data-points per patient) collected under unbalanced designs. Such a population approach will also allow therapeutic drug monitoring of ifosfamide, which can help in further tailoring ifosfamide treatment to the specific needs of the individual patient.
The aims of the research described in this thesis were to develop bioanalytical methods for determination of ifosfamide and its metabolites, to build population pharmacokinetic models for these compounds and to apply thee techniques in various clinical studies on ifosfamide.

Bioanalysis of ifosfamide and metabolites

Assessment of the pharmacokinetics of ifosfamide and metabolites has long been impaired by the lack of reliable bioanalytical assays. The availability of high through-put bioanalytical methods for all relevant metabolites of ifosfamide is a prerequisite for the pharmacological evaluation of the drug in cancer patients. In chapter 2 several new analytical methods are described for the determination of ifosfamide and its main metabolites. Gas chromatography was used to determine ifosfamide and its deactivated metabolites (chapter 2.1). Knowledge of the pharmacokinetics of the deactivated metabolites 2- and 3-dechloroethylifosfamide is crucial for insight into the risk of central neurotoxicity during ifosfamide treatment. In a

comparison between nitrogen-phosphorous detection and positive ion electron-impact ion-trap mass spectrometry, the former proved to be superior in sensitivity, accuracy and precision. The main activated metabolites 4-hydroxyifosfamide (chapter 2.2) and ifosforamide mustard (chapter 2.3) were determined using two high-performance liquid chromatography assays. The limitation of the highly unstable character of these metabolites was addressed by derivatization creating stabile, UV-absorbing derivatives. All analytical methods proved to be specific, sensitive, accurate and precise, and could be employed in the analysis of patient samples in a hospital setting.
The choice of the matrix for the pharmacokinetic assessment might be of importance, as literature previously indicated possible differences in metabolite distribution between plasma and erythrocytes. Consequently, drug concentration-time profiles in whole blood and plasma could differ, thereby yielding different values for the pharmacokinetic parameters. These data led us to investigate the *in vitro* and *in vivo* distribution of ifosfamide and its metabolites. These studies indicated that ifosfamide and its metabolites rapidly reach distribution equilibrium between erythrocytes and plasma, with ifosforamide mustard being the slowest (chapter 2.4). A strong parallelism in the erythrocyte and plasma concentration profiles was observed for all compounds, which indicates that no differences will arise in the assessment of pharmacokinetic parameters using either matrix. Thus, pharmacokinetic assessment using only plasma sampling can yield direct, accurate and relevant relationships with efficacy and toxicity of ifosfamide treated patients.

Development of population pharmacokinetic models

The development of improved bioanalytical assays allowed extensive pharmacokinetic assessment of ifosfamide and its metabolites. Pharmacokinetic assessment can identify key issues like population differences in pharmacokinetic parameters and the influence of dose and schedule of administration. A review of clinical pharmacokinetic studies on ifosfamide demonstrates that in most studies autoinduction has been observed (chapter 1). Although the mechanism of autoinduction is currently not completely understood, this phenomenon needed to be taken into account when establishing a pharmacokinetic model for ifosfamide and its metabolites. Development of these models was accelerated by using rich data populations. In a population of 15 patients with soft tissue sarcoma who received a 72-hour continuous infusion of ifosfamide, a non-linear population pharmacokinetic model was developed using non-linear mixed effect modelling (NONMEM). This model allowed quantification of the effect of autoinduction on the concentration-time profiles of ifosfamide with correlations between the ifosfamide plasma concentrations and the extent of the autoinduction (chapter 3.1).
In order to determine whether this population pharmacokinetic model was adequate in describing the pharmacokinetics of ifosfamide, a comparison was made with two other structural models: one without autoinduction and one with a time-dependent development of autoinduction of ifosfamide (chapter 3.2). The comparison was made in a population of 14 patients with small cell lung cancer who received a 1-hour intravenous infusion of ifosfamide daily for one or two days in combination with paclitaxel and carboplatin. Again, the model presented in chapter 3.1 described the concentration-time profiles of ifosfamide best. The Bayesian estimates of the pharmacokinetic parameters were used to describe the

pharmacokinetics of 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide. Dose-fractionation over two days compared to one day resulted in increased metabolite formation, especially for 2-dechloroethylifosfamide, probably due to increased autoinduction.
This effect of schedule was investigated further in a population pharmacokinetic study of all quantifiable analytes (chapter 2) in 56 patients, who were divided in three groups according to the length of ifosfamide infusion (chapter 3.3). The rate by which the autoinduction developed and the fractions metabolized to 2- and 3-dechloroethyl-ifosfamide were found to be significantly dependent on the infusion schedule. The observed differences in the parameters were, however, comparable to their interindividual variability and were, therefore, considered to be of minor clinical importance. Autoinduction caused a less than proportional increase in the area under the ifosfamide plasma concentration-time curve (measurement of exposure) and more than proportional increase in metabolite exposure with increasing ifosfamide dose. This study demonstrated that the duration of ifosfamide infusion influences the exposure to the parent and its metabolite 3-dechloroethylifosfamide. The observed dose and infusion duration dependency should be taken into account when the pharmacodynamics of different infusion schedules are evaluated.

Clinical applications

The developed pharmacokinetic population models (chapter 3) were valuable tools in the determination of the pharmacokinetic profiles of ifosfamide and its metabolites in various clinical studies on ifosfamide (chapter 4). Responses to ifosfamide treatment and toxicities vary to a great degree among patients. This variability might be explained by differences in pharmacokinetics. Characterising covariates which contribute to the variation in the pharmacokinetics is therefore of paramount importance. Therefore, the pharmacokinetics, relations between the pharmacokinetics and covariates and pharmacodynamics of ifosfamide and 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide were assessed in a population of 20 patients with soft tissue sarcoma, who received ifosfamide administered as a 72-hour continuous intravenous infusion (chapter 4.1). The population pharmacokinetic model (according to chapter 3.1) was built in a sequential manner, starting with a covariate-free model and progressing to a covariate model with the aid of generalized additive modelling. The addition of the covariates weight, body surface area, albumin, serum creatinine, serum urea, alkaline phosphatase and lactate dehydrogenase improved the prediction errors of the model. Significant pharmacokinetic-pharmacodynamic relationships were observed between the exposure to 2- and 3-dechloroethylifosfamide and orientational disorder, a neurotoxic side-effect. No pharmacokinetic-pharmacodynamic relationships between exposure to 4-hydroxyifosfamide and haematological toxicities could be observed in this population.
Children are a special population within the field of oncology. Only limited information is available on the pharmacokinetics of ifosfamide and its metabolites in paediatric patients, due to the ethical problems involved in these studies. We assessed the feasibility of a sparse data approach for the determination of the population pharmacokinetics of ifosfamide, 2- and 3-dechloroethylifosfamide and 4-hydroxyifosfamide in 32 children treated with various schedules of single agent ifosfamide therapy against various malignant tumours (chapter 4.2). A non-linear population pharmacokinetic model with linear development of autoinduction

was implemented. In chapter 3.2 this model proved less accurate in the description of the ifosfamide concentration-time profiles than the model presented in chapters 3.1 and 3.3. However, the more elaborate ifosfamide concentration dependent autoinduction model could not be applied in this sparse data population. Cross-validation by bootstrapping the data indicated accurate description of the population pharmacokinetic parameter without bias.
Specific isoenzymes are responsible for ifosfamide metabolism; activation is mediated by CYP3A4 and deactivation by CYP3A4 and CYP2B6. Therefore, modulation of the metabolism of ifosfamide may lead to an improved efficacy/toxicity ratio. Modulation was investigated by co-administrating ketoconazole and rifampicin, a potent inhibitor of CYP3A4 and inducer of CYP3A4/2B6, respectively (chapter 4.3). In a double randomized, two-way cross-over study a total of 16 patients received ifosfamide either alone or in combination with ketoconazole or rifampicin. Pharmacokinetics were assessed using the models developed in chapter 3. Rifampicin increased metabolism of ifosfamide without specifically favouring the activation or deactivation route of ifosfamide. Ketoconazole decreased activation to 4-hydroxyifosfamide. Thus, disappointingly this pharmacokinetic study indicated that no therapeutic benefit may be gained by modulation of ifosfamide therapy with rifampicin or ketoconazole in humans.
Commonly, chemotherapeutic agents are applied in combination-schedules in order to maximize the efficacy. The novel combination of ifosfamide and the topoisomerase I inhibitor topotecan was investigated in a phase I trial (chapter 4.4). Preliminary results indicate that the combination of topotecan administered as a 30-minute infusion daily times 3 with ifosfamide administered as a 1-hour infusion daily times 3 every three weeks to patients with advanced malignancies was feasible. Haematological toxicities were dose limiting. The maximum tolerated dose of topotecan has not yet been reached. The pharmacokinetics of topotecan and ifosfamide and its metabolites were similar to those observed after single agent administration suggesting that the drugs did not interact pharmacokinetically. Sigmoidal E_{max} models could be fit to the relationship between the areas under the plasma concentration-time curve of topotecan lactone and total topotecan, and the decrease in absolute neutrophil count. Partial responses were documented in three patients with ovarian cancer. Possible clinical benefit of this combination needs to be evaluated in phase II/III studies.

Perspectives and final remarks

Although ifosfamide has successfully been used for over 30 years in the treatment of various malignant diseases, there is still a need for understanding its variability in success and failure of treatment. Now that satisfactory bioanalytical methods and population pharmacokinetic models have been developed, the underlying mechanisms of this varibility may be better understood and a superior ifosfamide therapy may be developed, tailored to dosing-requirements of the individual patient. Furthermore, as individuals differ from each other in their concentration-effect relationships, the implication of schedule dependence is that varying the administration pattern, for example the infusion duration, between individuals may prove to be as important as individualising the dose-size of ifosfamide.
One of the remaining issues in the clinical pharmacology of ifosfamide is the firm establishment of pharmacokinetic-pharmacodynamic relationships that provide enough information for precise predictions of the clinical outcome of therapy based on the

pharmacokinetics of ifosfamide. Only if this requirement is met the further development of therapeutic drug monitoring and dose individualization of ifosfamide treatment will be possible. This thesis is the first step in that direction, but many more pieces of the puzzle need to be put together to achieve complete understanding of the extent and limitations of the clinical activity and toxicity of ifosfamide therapy.

Samenvatting en conclusies

Introductie

Ifosfamide is een alkylerend middel, dat toegepast wordt bij verschillende maligne aandoeningen bij zowel volwassenen als kinderen. Het gebruik van ifosfamide kan echter gepaard gaan met hematologische, neurologische en nefrologische neveneffecten. Ifosfamide is zelf niet actief en dient eerst door het cytochroom P450 (CYP) te worden omgezet in 4-hydroxyifosfamide. Spontane intracellulaire degradatie van 4-hydroxyifosfamide levert de uiteindelijke alkylerende metaboliet: ifosforamide mosterd. Ifosforamide mosterd is in staat om covalent aan het DNA van de tumorcel te binden, resulterend in verstoring van de functies van het DNA en uiteindelijk celdood. Naast deze activatieroute wordt ifosfamide ook gedeactiveerd tot de metabolieten 2- and 3-dechloorethylifosfamide. Hierbij komt een equimolaire hoeveelheid neurotoxisch chlooracetaldehyde vrij. Het metabolisme van ifosfamide is autoinduceerbaar, hetgeen inhoudt dat het metabolisme toeneemt met toenemende ifosfamide blootstelling. In hoofdstuk 1 wordt een overzicht gegeven van de klinische farmacologie van ifosfamide en metabolieten.
Kennis van het verband tussen de plasmaconcentraties (farmacokinetiek) en het effect (farmacodynamiek) is van belang voor een rationele ontwikkeling en een veilig en effectief gebruik van een chemotherapeuticum. De effectiviteit en specifieke bijwerkingen van de behandeling met ifosfamide kunnen worden teruggevoerd op het uitgebreide metabolisme. De beschrijving van de farmacokinetiek van ifosfamide en de metabolieten kan helpen de nu nog onvoorspelbare succeskans van ifosfamidebehandeling te verklaren. Hiervoor zijn studies met relatief grote aantallen patiënten nodig om de farmacokinetisch-farmacodynamische relaties en de variabiliteit daarin in kaart te brengen. Praktische en ethische bezwaren verhinderen echter dat er uitgebreid farmacologisch onderzoek kan worden gedaan in relatief grote populaties van vaak ernstig zieke patiënten. Daarom is er een dringende behoefte aan een methodiek die met slechts beperkte experimentele data per patiënt (een zogenaamde “sparse data approach”), verzameld onder ongecontroleerde omstandigheden, toch valide uitspraken kan doen over de relevante farmacokinetische en farmacodynamische relaties.
Doelstellingen van het onderzoek beschreven in dit proefschrift waren de ontwikkeling van bioanalytische methodes voor de bepaling van ifosfamide en metabolieten, de ontwikkeling van populatie farmacokinetische modellen voor ifosfamide en metabolieten en het toepassen van de technieken in verschillende klinische studies.

Bioanalyse van ifosfamide en metabolieten

Bepaling van de farmacokinetiek van ifosfamide en metabolieten is lange tijd belemmerd door een gebrek aan betrouwbare bioanalytische methodes. Klinische evaluatie vereist dat in een korte tijd een grote hoeveelheid monsters kan worden bepaald. In het tweede hoofdstuk worden verschillende bioanalytische methodes gepresenteerd voor ifosfamide en haar belangrijkste metabolieten. Gaschromatografie werd gebruikt voor de bepaling van ifosfamide en de gedeactiveerde metabolieten 2- en 3-dechloorethylifosfamide (hoofdstuk 2.1). Kennis van de farmacokinetiek van de gedeactiveerde metabolieten is cruciaal in de vaststelling van

het risico op de ontwikkeling van neurotoxiciteit tijdens de behandeling met ifosfamide. In een vergelijking tussen selectieve stikstof-fosfordetectie en “positive ion electron-impact ion-trap” massaspectrometrie, bleek de eerste detectiemethode gevoeliger, nauwkeuriger en preciezer. De belangrijkste geactiveerde metabolieten 4-hydroxyifosfamide (hoofdstuk 2.2) en ifosforamide mosterd (hoofdstuk 2.3) werden bepaald met twee hoge-druk-vloeistofchromatografische methodes. De beperkingen in kwantificeerbaarheid van deze instabiele verbindingen werden overwonnen door een derivatiseringsstap in te bouwen, waardoor een stabiel, UV-absorberend derivaat ontstond. Validatie van de drie methodes wees uit dat ze specifiek, gevoelig, nauwkeurig, precies en toepasbaar zijn voor de analyse van patiënten-monsters.
Uit literatuurgegevens blijkt dat de keuze van de matrix waarin de farmacokinetiek bepaald wordt, mogelijkerwijs van belang kan zijn. Verschillen in de verdeling van de metabolieten tussen plasma en rode bloedcellen zouden als gevolg kunnen hebben dat de concentratie-tijd profielen van een therapeuticum verschillen in volbloed en plasma. Daarom onderzochten wij de *in vitro* en *in vivo* distributie van ifosfamide en metabolieten (hoofdstuk 2.4). Uit deze studies bleek dat ifosfamide en metabolieten zich snel verdelen tussen de rode bloedcellen en het plasma, waarbij ifosforamide mosterd de langzaamste bleek te zijn. De farmacokinetische profielen van alle verbindingen vertoonden sterk parallellisme in rode bloedcellen en plasma. Dit geeft aan dat er geen verschillen worden verwacht in de kwantificering van de farmacokinetische parameters met behulp van plasma of rode bloedcellen.

Ontwikkeling van populatie farmacokinetische modellen

De ontwikkeling van de bioanalytische assays stelt ons in staat om belangrijke onderwerpen te onderzoeken als populatieverschillen in farmacokinetiek en de invloed van het toedieningsschema. Uit de literatuur blijkt dat in de meeste farmacokinetische studies met ifosfamide het fenomeen autoinductie werd geobserveerd (hoofdstuk 1). Het werkingsmechanisme van autoinductie van ifosfamide is op dit moment nog niet volledig duidelijk. Door de grote invloed van dit fenomeen op de farmacokinetiek dient het echter wel opgenomen te worden in de ontwikkeling van een populatie farmacokinetisch model voor ifosfamide. De ontwikkeling van dit model kon versneld worden door gebruik te maken van groepen patiënten waarbij veel monsters per patiënt afgenomen waren. Dientengevolge werd een niet-lineair populatie farmacokinetisch model ontwikkeld in een groep van 15 patiënten met weke delen tumoren, die een 72-uurs continu infuus van ifosfamide ontvingen (hoofdstuk 3.1). Met dit model kon het effect van de autoinductie op de concentratie-tijd profielen van ifosfamide worden bepaald. Hierbij werd gebruik gemaakt van het programma “non-linear mixed effect modelling” (NONMEM). Het model beschreef de autoinductie door de mate van ontwikkeling van de autoinductie af te laten hangen van de hoogte van de ifosfamide concentraties.
Om de geschiktheid van dit model verder te valideren werd het vergeleken met twee andere modellen waarbij farmacokinetiek werd beschreven zonder autoinductie of met een tijdsafhankelijke ontwikkeling van autoinductie (hoofdstuk 3.2). De vergelijking werd gemaakt in een populatie van 14 patiënten met kleincellige longcarcinoom, die een 1-uurs intraveneus

infuus van ifosfamide ontvingen over 1 of 2 dagen in combinatie met paclitaxel en carboplatine. Opnieuw beschreef het model van hoofdstuk 3.1 de concentratie-tijd profielen van ifosfamide het beste. Bayesiaanse schattingen van de farmacokinetische parameters van ifosfamide werden gebruikt bij de beschrijving van de farmacokinetiek van 2- en 3-dechloorethylifosfamide en 4-hydroxyifosfamide. Fractionering van de ifosfamide dosis over 2 dagen in plaats van 1 dag resulteerde in toegenomen metabolietvorming. Dit was vooral het geval voor 2-dechloorethyl-ifosfamide. De toename kan verklaard worden door toegenomen autoinductie.
Het effect van het toedieningsschema werd verder onderzocht in een populatie farmacokinetische studie van alle kwantificeerbare verbindingen (hoofdstuk 2) in drie groepen van in totaal 56 patiënten (hoofdstuk 3.3). De groepen waren ingedeeld naar lengte van de infuusduur: 1-4 uur, 24-72 uur en 96-240 uur. De snelheid waarmee de autoinductie zich ontwikkelde en de fracties van ifosfamide die tot 2- en 3-dechloorethylifosfamide werden omgezet waren significant afhankelijk van de infuusduur. De relatie werd echter als minder klinisch relevant beoordeeld, omdat de verschillen in dezelfde orde van grootte lagen als de interindividuele variabiliteit in deze parameters. Autoinductie resulteerde in een minder dan proportionele toename in de oppervlakte onder de plasma concentratie-tijd curve (een maat voor blootstelling) van ifosfamide en een meer dan proportionele toename aan metaboliet blootstelling bij hogere ifosfamide doseringen. Deze studie toonde verder aan dat de duur van het ifosfamide infuus de blootstelling aan ifosfamide en 3-dechloorethylifosfamide beïnvloedt. Bij de bestudering van de farmacodynamiek van ifosfamide moet rekening worden gehouden met deze dosis- en infuusduurafhankelijkheid van de farmacokinetiek.

Klinische toepassingen

De ontwikkelde populatie farmacokinetische modellen (hoofdstuk 3) waren waardevolle technieken bij de bepaling van de farmacokinetische profielen van ifosfamide en metabolieten in verschillende klinische studies (hoofdstuk 4). De kans op respons op de behandeling met ifosfamide, maar ook de kans op ernstige bijwerkingen verschilt enorm per patiënt. Deze variabiliteit zou verklaard kunnen worden door verschillen in de farmacokinetiek. Het is daarom van belang om mogelijke covariaten in kaart te brengen, die bijdragen aan de variabiliteit in farmacokinetiek. Derhalve werden de farmacokinetiek, de relaties tussen farmacokinetiek en covariaten en farmacodynamiek van ifosfamide, 2- en 3-dechloorethylifosfamide en 4-hydroxyifosfamide bepaald in een populatie van 20 patiënten met weke delen tumoren, die een 72-uurs continue infuus van ifosfamide ontvingen (hoofdstuk 4.1). Het populatie farmacokinetisch model van hoofdstuk 3.1 werd verder uitgebreid door stapsgewijze toevoeging van covariaten tot een volledig covariatenmodel met behulp van de techniek “generalized additive modelling”. De toevoeging van de covariaten lichaamsgewicht, lichaamsoppervlakte, albumine, serum creatinine, serum ureum, alkalische fosfatase en lactaat dehydrogenase verbeterde de voorspelbaarheid van het model. Er werden significante farmacokinetisch-farmacodynamische relaties waargenomen tussen 2- en 3-dechloorethylifosfamide en oriëntatievermogen (een neurologische bijwerking). Er werden geen relaties gezien tussen 4-hydroxyifosfamide en de beenmergdepressie in deze populatie.

Kinderen zijn een aparte groep binnen de oncologie. Door de praktische en ethische bezwaren tegen uitgebreide farmacokinetische bemonstering, is er slechts beperkte kennis over de farmacokinetiek van ifosfamide en metabolieten in de pediatrische populatie. Deze kennis is zeer gewenst voor de optimalisering van de ifosfamidebehandeling bij kinderen. Daarom onderzochten wij de mogelijkheid van een "sparse data approach" in de bepaling van de populatie farmacokinetiek van ifosfamide, 2- en 3-dechloorethylifosfamide en 4-hydroxyifosfamide in 32 kinderen met verschillende typen maligne tumoren, die een grote verscheidenheid aan infuusschemata met ifosfamide ontvingen (hoofdstuk 4.2). Er werd een niet-lineair populatie farmacokinetisch model met een lineaire tijdafhankelijke ontwikkeling toegepast. Dit model bleek voorheen (hoofdstuk 3.2) inferieur aan een model met een ifosfamide concentratie-afhankelijke ontwikkeling van autoinductie (hoofdstuk 3.1). Dit laatste, meer complexe model kon echter niet worden gebruikt in deze populatie met beperkte informatie per individu. Kruisvalidatie met behulp van een zogenaamde "bootstrap" methode gaf aan dat de beschrijving van de farmacokinetische parameters adequaat en zonder bias was.

Specifieke isoenzymen zijn verantwoordelijk voor de verschillende routes in het metabolisme van ifosfamide; CYP3A4 is verantwoordelijk voor de activatie- en CYP3A4 en 2B6 voor de deactivatieroute. Specifieke modulatie van het metabolisme zou kunnen leiden tot een verbetering van de verhouding tussen de effectiviteit en bijwerkingen van de behandeling. Een farmacokinetische evaluatie kan inzicht verschaffen in mogelijke metabole verschuivingen als gevolg van de modulatie. Modulatie werd onderzocht met behulp van het CYP3A4-remmende ketoconazol en de CYP3A4/2B6-inducerende rifampicine in een dubbel gerandomiseerde, cross-over studie in 16 patiënten, die ifosfamide monotherapie of in combinatie met ketoconazol of rifampicine ontvingen. Rifampicine verhoogde het metabolisme van ifosfamide zonder onderscheid te maken tussen de activatie- en deactivatieroute. Ketoconazol daarentegen verlaagde de activatie tot 4-hydroxyifosfamide. Dit impliceert derhalve dat de toediening van ketoconazol of rifampicine aan de ifosfamide behandeling mogelijk niet zal leiden tot een therapeutisch voordeel.

Chemotherapeutica worden vaak gecombineerd om maximalisatie van de effectiviteit te bereiken. De nieuwe combinatie van ifosfamide met de topoisomerase I remmer topotecan werd onderzocht in een fase I studie (hoofdstuk 4.4). Uit voorlopige resultaten blijkt dat de combinatie van topotecan toegediend als 30-minuten infuus gedurende 3 dagen, met ifosfamide toegediend als 1-uurs infuus gedurende dezelfde 3 dagen, aan patiënten met vergevorderde maligniteiten haalbaar was. Hematologische bijwerkingen bleken dosis-limiterend te zijn. De maximaal verdraagbare dosering van topotecan is echter nog niet vastgesteld. De farmacokinetiek van topotecan, ifosfamide en metabolieten was gelijk aan de farmacokinetiek na enkelvoudige toediening. Dit duidde op de afwezigheid van een farmacokinetische interactie van deze combinatie. De relatie tussen de blootstelling aan lacton en totaal topotecan en de afname in neutrofielen kon beschreven worden met sigmoïdale E_{max} modellen. Partiële responsen werden geobserveerd in drie patiënten met een ovariumcarcinoom. Mogelijke klinisch voordeel van deze combinatie dient nader te worden onderzocht in fase II en III studies.

Perspectieven en afsluitende opmerkingen

Ondanks dat ifosfamide al meer dan dertig jaar succesvol wordt ingezet tegen verscheidene maligne aandoeningen, is er nog steeds behoefte aan kennis over de variabiliteit van het slagen of mislukken van de behandeling. Nu er afdoende bioanalytische methoden en populatie farmacokinetische modellen beschikbaar zijn kunnen de onderliggende mechanismen van deze variabiliteit beter begrepen worden. Dit kan resulteren in een verbeterde therapie van ifosfamide, die beter aansluit op de specifiek doseringsbehoefte van de individuele patiënt. Omdat patiënten verschillen in hun concentratie-effectrelaties, zullen de implicaties van de schema-afhankelijkheid van het metabolisme van ifosfamide misschien even belangrijk worden als de absolute doseringshoeveelheid.
Op dit moment zijn de farmacokinetisch-farmacodynamische relaties van ifosfamide en metabolieten nog niet onomstotelijk vastgesteld. Daarnaast zullen deze relaties ook een voldoende mate van voorspelbaarheid moeten bevatten. Alleen dan kan de geneesmiddelbewaking en dosisindividualisatie van ifosfamide verder worden ontwikkeld. Dit proefschrift is de eerste stap in die richting, maar er zijn er nog vele nodig om alle mogelijkheden en onmogelijkheden van de behandeling met ifosfamide te begrijpen.

Het proefschrift: een eenvoudig verhaal

Ifosfamide is een antikankermiddel dat reeds sinds de jaren ‘70 succesvol wordt ingezet in de strijd tegen diverse vormen van kanker bij zowel volwassenen als kinderen. Remming van de aanmaak van bloedcellen en bijwerkingen op het centrale zenuwstelsel en de nieren blijven echter ernstig beperkende factoren van de behandeling met ifosfamide. Het belang van een goede dosering is groot. Overdosering kan leiden tot ernstige bijwerkingen, maar onderdosering kan leiden tot falen van de therapie. De balans tussen bijwerkingen en succesvolle behandeling is moeilijk te bepalen en verschilt tussen patiënten enorm. Deze verschillen tussen de patiënten zijn deels te verklaren door verschillen in afbraak van het geneesmiddel in het lichaam. De ene patiënt breekt een geneesmiddel sneller af dan de andere patiënt. De verschillende afbraakprodukten van ifosfamide zijn verantwoordelijk voor de ernstige bijwerkingen, maar ook voor het kankerceldodend effect. Kennis van de afbraak van ifosfamide in het lichaam is van belang om tot een beter begrip te komen van de effecten van het kankerceldodend middel in het lichaam. Dit kan door zogenaamde bloedspiegel-effectrelaties te beschrijven. Deze relaties kunnen de behandeld arts in staat stellen om aan de hand van de bloedspiegels van ifosfamide, uitspraken te doen over de te verwachten bijwerkingen, maar ook het mogelijke slagen van de therapie. Op basis van de bloedspiegels kan de dosis en het behandelschema meer op de individuele patiënt worden toegespitst. Het afnemen van veel bloedmonsters om deze relaties te beschrijven stuit echter op ethische bezwaren, want het is belastend en vermindert de kwaliteit van leven van de patiënt.

In dit proefschrift zijn nieuwe methoden beschreven voor het meten en beschrijven van het bloedspiegelverloop van ifosfamide en metabolieten. Deze technieken zijn vervolgens gebruikt om o.a. het effect van de dosis en duur van het infuus, patiënteigenschappen als gewicht, lichaamsgrootte, lever- en nierfunctie en het effect van andere geneesmiddelen op het bloedspiegelverloop van ifosfamide en metabolieten vast te stellen. Het bloedspiegel-verloop in kinderen is vastgesteld met behulp van een zogenaamde groepsbenadering, waarbij slechts enkele bloedafnames per kind nodig waren. De optimale dosering van een combinatie van ifosfamide met een ander cytostaticum is onderzocht. De verworven kennis van het bloedspiegelverloop van ifosfamide en metabolieten kan bijdragen tot een beter op maat gemaakte therapie voor kankerpatiënten met minder kans op ernstige bijwerkingen en meer kans op een succesvolle behandeling.

Curriculum Vitae

Thomas Kerbusch werd geboren op 30 april 1972 te Nijmegen. In 1990 behaalde hij het Atheneum diploma aan het Duckenburg College te Nijmegen. In datzelfde jaar werd een aanvang gemaakt met de studie Biologie aan de Rijksuniversiteit te Leiden. Na het behalen van het propaedeutisch examen in 1991 startte hij met de doctoraalstudie Bio-Farmaceutische Wetenschappen, tevens aan de Rijksuniversiteit te Leiden. In het kader van de hoofdvakstage bij de vakgroep Farmacologie werd van januari 1995 tot januari 1996 onderzoek gedaan naar de relaties tussen de farmacokinetiek en farmacodynamiek van synthetische opiaten in de rat. De studie werd afgerond met een onderzoeksstage van januari tot augustus 1996 bij de Division of Clinical Pharmacology van de Georgetown University Medical Center, Washington DC, VS. Alwaar de interactie tussen het neurolepticum pimozide en het antibioticum clarithromycine in gezonde vrijwilligers werd onderzocht. Vanaf november 1996 was hij als onderzoeker-in-opleiding werkzaam bij de secties Medische Oncologie, en Farmacie en Farmacologie van het Nederland Kanker Instituut / Antoni van Leeuwenhoek Ziekenhuis te Amsterdam, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Tegelijkertijd volgde hij de opleiding ter verkrijging van de aantekening ‘klinisch farmacoloog’ onder auspiciën van de Nederlandse Vereniging voor Klinische Farmacologie en Biofarmacie. Thomas Kerbusch is momenteel werkzaam als postdoctoraal onderzoeker aan de Universiteit van Uppsala in Zweden.

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