Pharmacological Studies of Ifosfamide

Martin Steven HIGHLEY

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Declaration of originality

I declare that this thesis was composed by myself, and with the exception below, the work here presented is entirely my own.

Signature

The analysis of ifosfamide and its metabolites, described in chapter 5, was performed with Dr G Momerency, Laboratory for Cancer Research and Clinical Oncology, University of Antwerp, Belgium.

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Highley, M.S. and De Bruijn, E.A. (1996). Erythrocytes and the transport of drugs and endogenous compounds. *Pharm Res*, **13**, 186-95.

Highley, M.S., Schrijvers, D., Van Oosterom, A.T., Harper, P.G., Momerency, G., Van Cauwenberghe, K., Maes, R.A.A., De Bruijn, E.A., and Edelstein, M.B. (1997). Activated oxazaphosphorines are transported predominantly by erythrocytes. *Ann Oncol*, **8**, 1139-44.

Momerency, G., Van Cauwenberghe, K., De Bruijn, E.A., Van Oosterom, A.T., Highley, M.S., and Harper, P.G. (1994). Determination of iphosphamide and seven metabolites in blood plasma, as stable trifluoroacetyl derivatives by electron capture chemical ionisation GC-MS. *J High Res Chromatogr*, **17**, 655-61.

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Abstract

Ifosfamide is a well established oxazaphosphorine alkylating agent used in the treatment of many malignancies. It is a pro-drug, requiring activation, mainly via the hepatic cytochrome mixed function oxidase P-450 system, and 4-hydroxylation leads to the active compound isophosphoramide mustard. Encephalopathy of varying degrees, usually somnolence, occurs in approximately 10% of patients treated with intravenous ifosfamide. The development of an oral formulation has been hindered by a higher incidence of 50%. Pharmacokinetic processes are probably central to the encephalopathy; the chemical entity responsible has not yet been firmly identified, but it is likely to be a metabolite of ifosfamide, with chloroacetaldehyde as the main suspect. Pharmacokinetic investigations have been aided recently by the pairing of analytical techniques, allowing the study of separate metabolites. It is also becoming clear that the erythrocyte is a significant carrier of substances in the circulation and should be considered in such studies. This thesis investigates the plasma and erythrocyte pharmacokinetics of ifosfamide, and neurotoxicity after oral administration.

The plasma pharmacokinetics and neurotoxicity of oral ifosfamide were studied in seven females with carcinoma of the cervix and four males with non-small cell lung cancer. The median age was 58 years (range 33 – 70). Oral ifosfamide was administered at a dose of 500 mg twice daily for 14 days; 300 mg of mesna was given twice daily concurrently for uroprotection. Gas chromatography – mass spectrometry was used to analyse ifosfamide and seven of its metabolites; isophosphoramide mustard, 2- and 3- dechloroethylifosfamide, carboxyifosfamide, ketoifosfamide, chloroethylamine, and 1, 3-oxazolidine-2-one. The basic metabolites chloroethylamine and 1, 3-oxazolidine-2-one, predicted from the metabolism of cyclophosphamide, have not been described previously in biological fluids. Ifosfamide neurotoxicity was assessed using the electroencephalogram, psychometric testing, the national adult reading test, the mini-mental state examination, and clinical evaluation of symptoms. The mean Cmax, and AUC to eight hours, of

ifosfamide were less on day 14, but not significantly so. Ifosfamide Cmax was 53.1 μM (SD ± 18.5) on day 1, and 50.7 μM (SD ± 25.8) on day 14. Corresponding values for the AUC to eight hours were 293 μM . hr (SD ± 67), and 256 μM . hr (SD ± 118). It was not possible to estimate the AUC to eight hours accurately, on account of the twice daily dosage regimen. The mean absorption rate constant of ifosfamide on day 1 was 1.23 hr^{-1} (SD ± 1.01). The Cmax of most ifosfamide metabolites increased on day 14, particularly ketoifosfamide, 2- and 3- dechloroethylifosfamide, carboxyifosfamide and 1, 3-oxazolidine-2-one, whereas that of isophosphoramide mustard was unchanged. Decreased alpha frequency was seen in the electroencephalogram, with the development of pathological slow wave activity. Psychometric performance was also impaired. All patients developed a feeling of detachment, or an inability to concentrate, and two suffered hallucinations.

The plasma and erythrocyte pharmacokinetics of ifosfamide and its metabolites were studied in five patients receiving intravenous ifosfamide. Using the newly developed MESED instrument, a direct determination of red cell associated compounds was performed on unwashed erythrocytes, maintained in their natural environment, without disruption of the normal equilibrium existing between red cells and plasma in whole blood. The mean ratio of erythrocyte AUC to plasma AUC was greater than unity for ifosfamide and all metabolites. The difference between the AUCs was most evident for carboxyifosfamide, isophosphoramide mustard, and ifosfamide.

In conclusion, oral ifosfamide 500 mg twice daily for 14 days is too neurotoxic for routine use, with impairment of psychometric performance and electroencephalographic appearances. The pharmacokinetic data support the hypothesis that dechloroethylation is important in the generation of neurotoxicity. Ifosfamide and its metabolites enter the erythrocyte compartment freely, and carboxyifosfamide and isophosphoramide mustard are concentrated most significantly within this fraction.

Abbreviations

AUC	Area under the concentration – time curve
AUC_E	Area under the erythrocyte concentration – time curve
AUC_t	Area under the concentration – time curve, to the last sampling time
AUC_P	Area under the plasma concentration – time curve
AUC_8	Area under the concentration – time curve, to eight hours
AUC_∞	Area under the concentration – time curve, extrapolated to infinity
AUMC	Area under the first moment curve
CEA	Chloroethylamine
CEOX	N-chloroethyl-1, 3-oxazolidine-2-one
CI–	Negative Chemical Ionisation
CIPA	Carboxyifosfamide
CL	Clearance
Cmax	Maximum concentration
Cmin	Minimum concentration
CNS	Central Nervous System
CPA	Cyclophosphamide
CPM	Phosphoramide Mustard
CSF	Cerebrospinal Fluid
Css	Average drug concentration
CTC	Common Toxicity Criteria
2-DCEI	2-Dechloroethylifosfamide
3-DCEI	3-Dechloroethylifosfamide
EDTA	Ethylenediaminetetra-acetic acid
EEG	Electroencephalogram
EI+	Electron Impact Ionisation
GC	Gas Chromatography
GC–MS	Gas Chromatography – Mass Spectrometry
HPLC	High Performance Liquid Chromatography

IPA	Ifosfamide
IPM	Isophosphoramide Mustard
IQ	Intelligence Quotient
IS	Internal Standard
KA	Absorption rate constant
KIPA	Ketoifosfamide
k	Rate constant
LC–MS	Liquid Chromatography – Mass Spectrometry
MAP	Maximum a posteriori probability
MESED	Measurement of Sediment
MIM	Multiple Ion Monitoring
MMS	Mini Mental State examination
MRT	Mean Residence Time
NART	National Adult Reading Test
NBP	Nitrobenzylpyridine
NMR	Nuclear Magnetic Resonance
NNM	Nor Nitrogen Mustard
NONMEM	Nonlinear mixed effect modelling
NSCLC	Non Small Cell Lung Cancer
OXA	1, 3-Oxazolidine-2-one
PD	Pharmacodynamic
PK	Pharmacokinetic
SCLC	Small Cell Lung Cancer
SIM	Single Ion Monitoring
$T_{1/2}$	Half-life
TFAA	Trifluoroacetic anhydride
TLC	Thin Layer Chromatography
Tmax	Time of maximum concentration
T_R	Retention time
Vd	Apparent volume of distribution
Vss	Apparent volume of distribution at the steady state
WHO	World Health Organisation

Contents

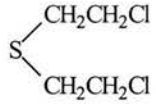
PART I INTRODUCTION AND GENERAL BACKGROUND

Chapter 1

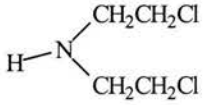
General Introduction

1.1 The development of the alkylating agents and oxazaphosphorines

The alkylating agents are the oldest class of anticancer drugs. The first alkylator, sulphur mustard (dichloroethyl sulphide), was synthesised in 1822 by Despretz (figure 1.1), and its toxic and vesicant properties described by Meyer in 1887 (Dacre and Goldman 1996).



Sulphur Mustard



Nitrogen Mustard

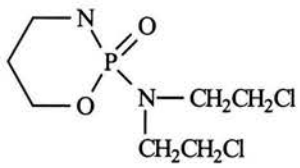
Figure 1.1. The structures of sulphur and nitrogen mustards.

Sulphur mustard was used as an agent of chemical warfare for the first time at Passchendale in July 1917. The predicted effects on the lungs, eyes and skin were observed, but in addition unexpected bone marrow aplasia, destruction of lymphoid tissue, and gastrointestinal ulceration were noted (Krumbaar and Krumbaar 1919). During the second world war the biological and chemical properties of nitrogen mustard [methyl bis - (2-chloroethyl)-amine] were investigated. The studies of Gilman and

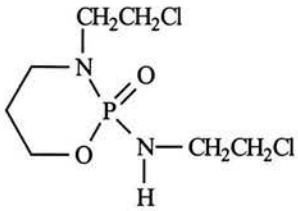
colleagues, using transplanted lymphosarcomas in mice, confirmed the cytotoxic action of nitrogen mustard on lymphoid tissue, and the first clinical trials in 1942 marked the dawn of modern cancer chemotherapy. In 1945 nitrogen mustard was declassified, and details of its successful use in Hodgkins disease were published (Gilman and Philips 1946; Goodman et al 1946).

Nitrogen mustard has a narrow therapeutic index. Alkylating agents are not tumour selective and are cytotoxic by virtue of their ability to alkylate nucleophilic centres in the DNA molecule; the potential to damage both tumour and normal host tissues was appreciated early in their development. In 1952 Druckrey and Raabe suggested that the administration of a highly reactive drug as a chemically masked inactive "transport form", rather than an active compound, was a possibility; it was envisaged that transformation into the active form would occur in the body, ideally in tumour cells (Druckrey and Raabe 1952). This concept was termed "drug latentiation" in 1959 (Harper 1959), and has been influential in the development of more selective antineoplastic agents.

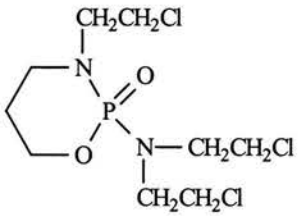
Inactivation of the highly reactive nitrogen mustard by chemical coupling is necessary for the formation of a well tolerated transport form. The biological action of the nitrogen mustards is dependent on the reactivity of the two functional chloroethylamino groups, which in turn is related to the basicity of the nitrogen atoms. Substituting electrophilic groups at the amino nitrogen reduces basicity, and therefore the reactivity of the functional groups. Conversely, introducing a nucleophilic group increases the reactivity. Arnold and Bourseaux (1958) coupled the mustard group to the electrophilic phosphoryl group, assuming that the resulting stable oxazaphosphorine would be the inactive transport form, which would fragment in vivo, releasing the active amine. Cyclophosphamide was the result (figure 1.2).



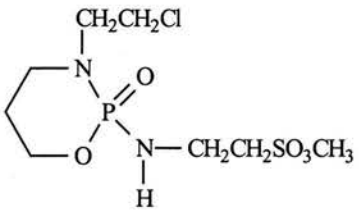
Cyclophosphamide



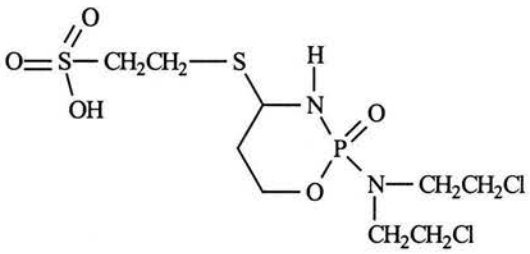
Ifosfamide



Trofosfamide



Sufosfamide



Mafosfamide

Figure 1.2. The structures of cyclophosphamide, ifosfamide, trofosfamide, sufosfamide and mafosfamide.

Since 1958, more than one thousand compounds have been synthesised, and in addition to cyclophosphamide four possess particularly favourable properties: trofosfamide, first seen in clinical practice in 1972; ifosfamide, in routine use since 1977; sufosfamide, a mixed function oxazaphosphorine with immunological properties; and mafosfamide, a stabilised primary oxazaphosphorine metabolite developed in the 1980s, but excluded from clinical practice by the development of local tissue irritation after intravenous administration. Ifosfamide is a structural isomer of cyclophosphamide, with one of the 2-chloroethyl groups positioned on the endocyclic nitrogen atom.

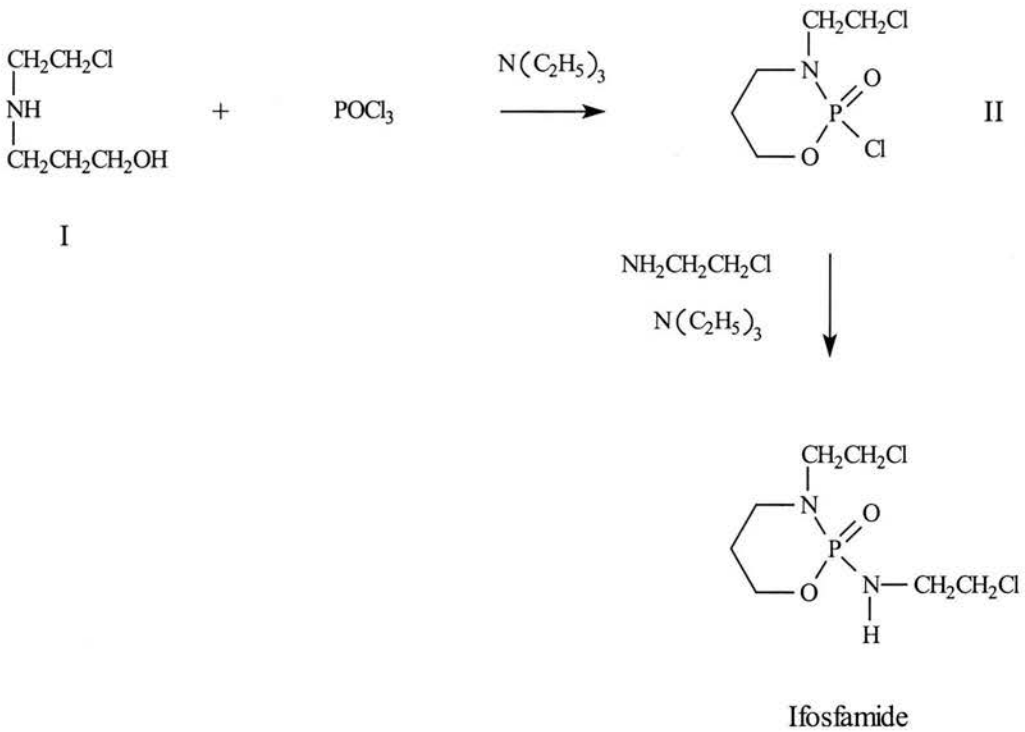


Figure 1.3. The synthesis of ifosfamide.

1.2 Synthesis and physical chemistry of ifosfamide

The synthesis of ifosfamide (3-(2-chloroethyl)-2-(2-chloroethylamino) tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide) is shown in figure 1.3. N-(β-chloroethyl)-N-(3-hydroxypropyl)amine (I) reacts with phosphorus oxychloride, yielding the oxazaphosphorine derivative II, which is then alkylated with N-β-chloroethylamine hydrochloride in the presence of triethylamine. Ifosfamide is a white crystalline powder, weakly hygroscopic, with a molecular weight of 261.08, and a melting point of 48°C to 51°C. It is 10% soluble in water (w/v). The pH of an unbuffered 5% solution of ifosfamide is 5.5. Ifosfamide is quite soluble in ethanol, chloroform and ethyl acetate, but only slightly soluble in ether.

1.3 The clinical use of ifosfamide

Ifosfamide was introduced into early clinical trials in 1972, as a less myelosuppressive alternative to cyclophosphamide. It has since become established in the treatment of sarcoma, lung cancer, cervical cancer, breast cancer, and also has activity in ovarian cancer, teratoma and lymphoma. Ifosfamide is used more frequently than cyclophosphamide in non-small cell lung cancer (NSCLC), where it is often combined with mitomycin C and cisplatin (Cullen et al 1988), in sarcomas, and in cervical cancer.

Ifosfamide generates response rates of greater than 20% in soft tissue sarcoma (Keohan and Taub 1997). A dose of 5 g m^{-2} has been shown to be more effective than cyclophosphamide given at myelosuppressive doses (1.5 g m^{-2}). In patients previously treated with doxorubicin containing regimens, ifosfamide given as a single agent has resulted in response rates of 8% to 38%, and it is presently the most active single salvage agent.

Haemorrhagic cystitis was the initial dose limiting toxicity of ifosfamide (Cox 1979), a problem which was overcome with the introduction of mesna (sodium-2-mercapto-

ethane sulphonate) in 1983. Myelosuppression is currently the main adverse effect. The white blood cell count nadir usually occurs between days 8 and 13 of the treatment cycle. Thrombocytopenia is seen less frequently, and is usually of mild to moderate severity. The bone marrow recovers completely by day 17 or 18.

Following the concomitant use of mesna, macrohaematuria is reported now in fewer than 5% of patients. However nephrotoxicity, in the form of glomerular or tubular dysfunction, remains a potential problem, particularly in paediatric patients. Nausea and vomiting are quite common, dose-related, and usually of moderate intensity. Alopecia occurs in almost all patients. Neurotoxicity, varying in severity from somnolence to severe encephalopathy and coma, can occur in 10% to 20% of patients receiving intravenous ifosfamide, and in up to 50% of patients treated orally.

1.4 The aims of the thesis

Ifosfamide is now well established in the treatment of malignant disease. However, side effects continue to restrict its use. Ifosfamide is a pro-drug, requiring activation to an active compound, a process which also generates toxic metabolites. The cystitis, and probably the neurotoxicity, are a result of this metabolic transformation, a complex and necessary process. Although the cystitis has been eliminated by the use of mesna, neurotoxicity has hindered the development of an oral formulation. The neurotoxic agent has not yet been positively identified, and it is possible that a different profile of metabolites is produced by the oral compared to the intravenous route. A more complete understanding of ifosfamide metabolism and pharmacokinetics is required. Furthermore, work to date has investigated the plasma compartment, but neglected the red cell compartment. The erythrocyte fraction can be important in the transfer of many compounds in the circulation (Driessen et al 1989a; 1989b; 1989c), and data is required on the red cell pharmacokinetics of ifosfamide and its metabolites.

The aims of the thesis are to:-

a Investigate the pharmacokinetics of oral ifosfamide

b Assess the neurotoxicity of oral ifosfamide

c Explore relationships between pharmacokinetic indices, and the development of neurotoxicity, following oral ifosfamide; in particular, the hypothesis that neurotoxicity is caused by the products of dechloroethylation

d Investigate the uptake of ifosfamide and its metabolites by erythrocytes

Chapter 2

The Pharmacokinetics of Ifosfamide

2.1 Introduction

Pharmacokinetic processes are those by which the body influences a drug, and comprise absorption, distribution, metabolism and excretion. Absorption occurs most commonly from the gastrointestinal tract after oral administration, although other modes of entry, such as the intramuscular and subcutaneous routes, are also used. Transfer of a drug from the site of absorption to the blood is followed by rapid distribution between the plasma, plasma proteins (usually albumin, but sometimes alpha-1-acid glycoprotein and occasionally globulin), plasma water and erythrocytes. As most drugs are relatively small molecules, they cross capillaries easily and reach the extracellular fluid of almost every organ in the body. The majority of drugs are also lipid soluble enough to cross cell membranes and distribute in the intracellular fluid. This transfer of drugs from blood to extravascular fluids (ie extracellular and intracellular water) and tissues is called distribution, which is usually a rapid and reversible process. The volume of distribution (Vd) is the volume into which a drug appears to distribute. Soon after intravenous injection, there is a distribution equilibrium between drug in the plasma and that in erythrocytes, other body fluids, and tissues. As a consequence of this dynamic equilibrium, changes in the concentration of drug in the plasma are taken to indicate concentration changes in other tissues. The initial rapid fall in drug levels after intravenous bolus injection, the distribution-elimination phase, is characterised by the α half-life, whereas the half-life after distribution, the terminal half-life, is denoted the β half-life.

Metabolism is the biochemical transformation of a drug in the tissues or plasma to metabolic products, and the transfer of a drug from the blood to urine, or other excretory compartments (eg bile, saliva or milk) is the process of excretion. Metabolism

and excretion are usually irreversible and together comprise drug elimination. Elimination processes are responsible for the physical or biochemical removal of a drug from the body. Pharmacokinetic equations are described in chapter 6.

Ifosfamide is a pro-drug, and therefore the metabolic process is central to its action, an extensive and diverse series of transformations occurring which are not fully understood. The study of pharmacokinetics is influenced by available analytical methodology. The plasma nitrobenzylpyridine (NBP) alkylating activity was the earliest method of measuring ifosfamide metabolites, based on the NBP reaction (chapter 5), and advances in the analysis of ifosfamide and its metabolites have preceded the gathering of more complete pharmacokinetic data.

2.2 Absorption

Oral ifosfamide is absorbed relatively quickly with peak levels occurring within one hour (Wagner and Drings 1986). Bioavailability studies have confirmed the extensive absorption of oral ifosfamide, with values of 90.5% and 107% for doses of 1 g and 2 g respectively (Cerny et al 1986). In a cross over study of oral and intravenous ifosfamide, twelve patients received 1 g m^{-2} and six patients 2 g m^{-2}, both groups crossing over on day 3 (Wagner and Drings 1986). The average bioavailability was 100%; induction of metabolism caused variations in the area under the plasma concentration-time curve (AUC), which were dependent on the sequence of administration. A mean bioavailability of 92% was reported by Kurowski et al (1991), and overall, these results indicate that ifosfamide is probably totally absorbed from the gastrointestinal tract. The high bioavailability of ifosfamide implies an insignificant first pass effect, even though ifosfamide is metabolized mainly by the hepatic cytochrome mixed function oxidase P-450 system (Allen et al 1976). From the intravenous dose, AUC and assumed liver blood flow, a theoretical maximum first pass effect of 5% to 6% has been calculated (Wagner and Drings 1986). Ifosfamide is also well absorbed when given subcutaneously, with a bioavailability of 90% to 100% (Cerny et al 1990b).

The small first pass effect is not consistent with the increased incidence of neurotoxicity seen after oral administration. Neurotoxicity is thought to be caused by a metabolite of the dechloroethylation pathway, probably chloroacetaldehyde. A low first pass effect suggests the generation of a small amount of a very toxic metabolite. It is possible that different profiles of oxazaphosphorine metabolites are produced after oral administration compared to the intravenous route, even though the extent of metabolism is similar, with a negligible first pass effect. In the same patient, Juma et al (1979) found higher levels of alkylating activity in serum after oral cyclophosphamide compared to intravenous dosing; the oral biovailability was nearly 100%. Similarly, in the study of Kurowski et al (1991), comparing oral and intravenous ifosfamide in the same patient (one dose on day 1, the other on day 3), the concentrations of activated ifosfamide were always higher after oral compared to intravenous administration. However, although the average oral bioavailability was 92%, when ifosfamide was given intravenously on day 3, a biovailability of more than 100% was calculated, whereas oral administration on day 3 led to a lower bioavailability.

2.3 Distribution

Ifosfamide is distributed mainly in body water, and numerous studies have reported a volume of distribution of about 38 L (Cerny et al 1986; Lind et al 1989a, 1989b; McNeil and Morgan 1981). Lewis et al (1990) reported median values of the same order for days 1 and 5 of a fractionated regimen (635 – 721 ml kg^{-1}). The volume of distribution of ifosfamide is approximately the sum of the vascular and extracellular spaces, suggesting a lack of binding to plasma and tissue proteins. Similar results have been obtained following oral ifosfamide administration, with values of 0.76 L kg^{-1} and 0.72 L kg^{-1} after 1 g m^{-2} and 2 g m^{-2} respectively (Wagner and Drings 1986). The metabolites of ifosfamide, unlike the parent compound, bind to plasma proteins both in vitro and in vivo. The volume of distribution of radiolabelled ifosfamide metabolites is equal to that of the plasma space volume (5.1 L) indicating that only limited diffusion occurs out of the plasma, consistent with a high degree of protein

binding (Allen and Creaven 1975). Ifosfamide microsomal metabolites are known to bind covalently to microsomal proteins and bovine serum albumin (Allen and Creaven 1973).

2.4 Metabolism

Ifosfamide is a structural isomer of cyclophosphamide, and follows the same metabolic pathways, although these are quantitatively different (Norpoth 1976a; Wagner et al 1981). The oxazaphosphorines require activation, predominantly via the hepatic cytochrome mixed function oxidase P-450 system (Allen et al 1976). There are large interindividual differences in the activities of various members of the P-450 superfamilies on account of environmental and genetic factors, and this is reflected in wide interindividual variability in the extent of activation and inactivation (Boddy et al 1992a). The lungs are a minor site of oxazaphosphorine metabolism (Brock and Hohorst 1967; Hill et al 1973). The metabolism of ifosfamide is depicted in figure 2.1.

2.4.1 Activation of ifosfamide

Ifosfamide is activated to 4-hydroxyifosfamide, which exists in equilibrium with its tautermeric form aldoifosfamide. Aldoifosfamide spontaneously decomposes to form isophosphoramide mustard (IPM), the ultimate alkylating agent. Information on the identification and quantitative analysis of 4-hydroxyifosfamide in humans and animals is sparse (Arndt et al 1988; Ikeuchi and Amano 1985; Kurowski et al 1991; Kurowski and Wagner 1993; Wagner et al 1981; Wiedemann et al 1993), as it is extremely unstable in the blood, and relatively low peak plasma concentrations (1 μM – 5 μM) are generated following therapeutic doses of ifosfamide. The plasma profile parallels – but only reaches 1% – that of ifosfamide. Similarly, few pharmacokinetic data are available for IPM (Bryant et al 1980). IPM can be converted into chloroethylamine (CEA) as shown in figure 2.2; in the presence of bicarbonate, 1,

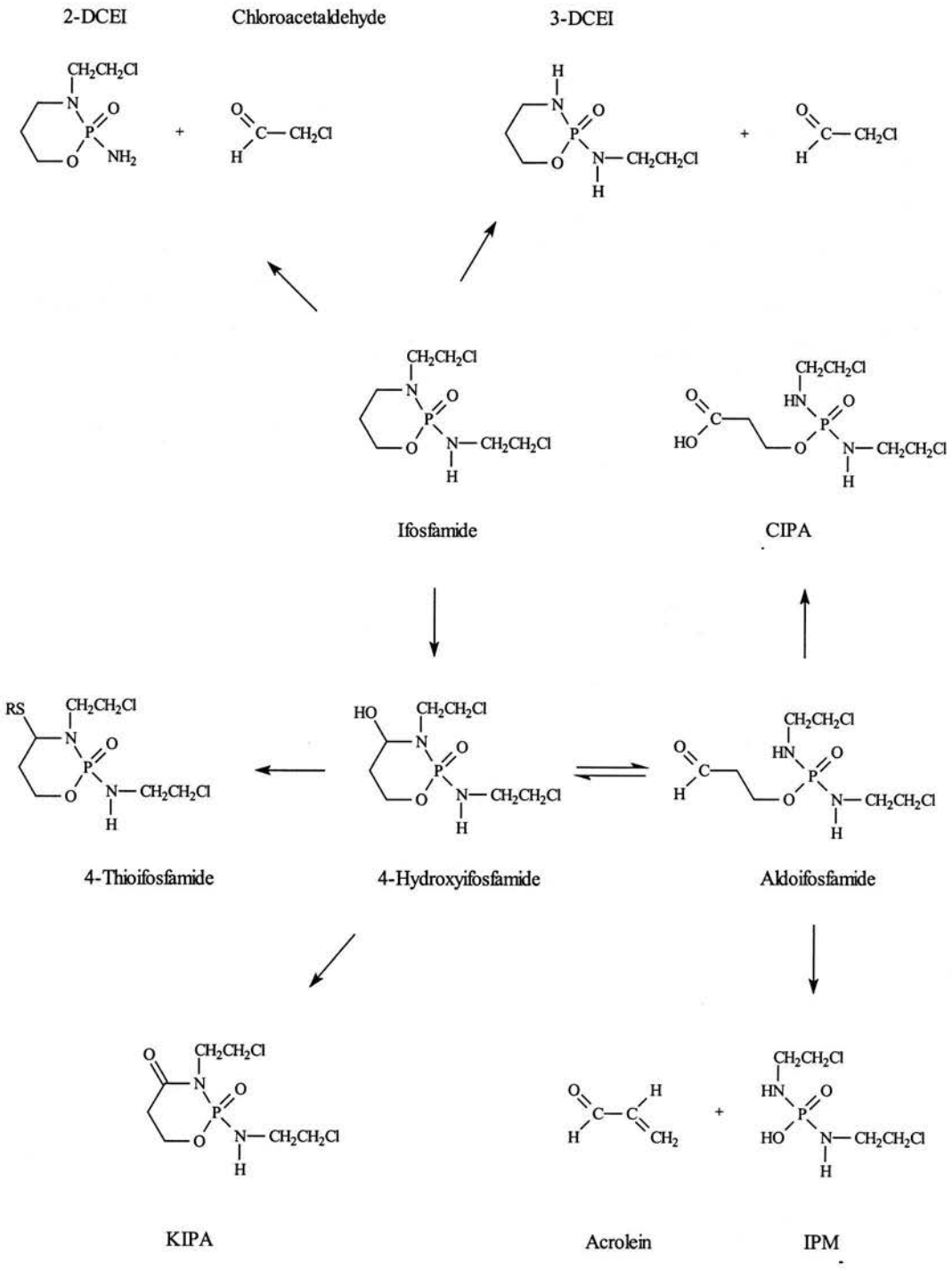


Figure 2.1. The metabolism of ifosfamide.

3-oxazolidine-2-one (OXA) is produced.

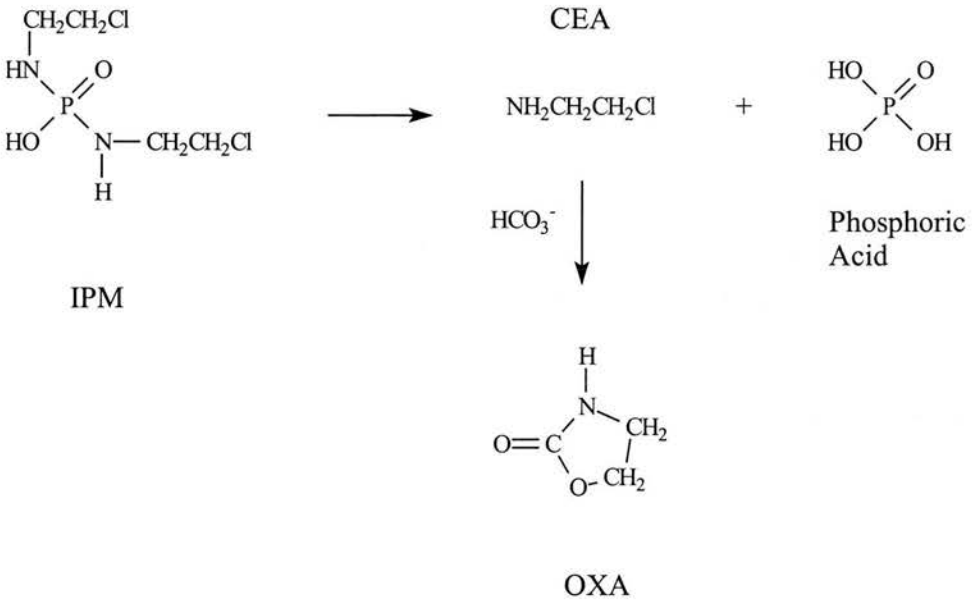


Figure 2.2. The conversion of IPM into CEA and OXA.

2.4.2 Acrolein

Acrolein is responsible for ifosfamide induced cystitis, which is routinely prevented by the concomitant administration of mesna. It is highly reactive with a short half-life, and is further metabolised to 3-hydroxypropyl mercapturic acid. Consequently the analysis of acrolein in plasma is difficult (Wagner et al 1981).

2.4.3 Carboxyifosfamide, ketoifosfamide and other by-products of activation

4-hydroxyifosfamide undergoes oxidation by an ALDH3 enzyme to carboxyifosfamide (CIPA), which has slight alkylating properties (Dockham et al 1992). There is a wide variation in the excretion of CIPA, possibly as a result of

phenotypic variation in ALDH activity (Goedde et al 1967). Boddy and co-workers found that the variability in plasma metabolite AUCs was greatest for CIPA, and it was postulated that this compound was formed from aldoifosfamide in the kidney and excreted without returning to the systemic circulation, or that plasma concentrations were below the limit of detection (Boddy et al 1993). Patients may be deficient in the ALDH enzyme systemically, but possess ALDH activity in the kidney. Phenotypic differences possibly influence efficacy and toxicity, with those patients possessing high aldehyde dehydrogenase activity less likely to respond to ifosfamide, whilst an increased susceptability to toxic effects occurs in those with low activity. Impaired excretion of carboxycyclophosphamide has been described, but not of CIPA (Boddy et al 1992a; Lind et al 1990b).

Ketoifosfamide (KIPA) is an inactive metabolite. Urinary excretion of KIPA increases over five days when ifosfamide is given in a fractionated manner, either intravenously or orally, but the urinary excretion of KIPA and CIPA is negligible, and it appears that the majority of activated ifosfamide is converted to IPM (Boddy et al 1993; Gilard et al 1993; Lind et al 1989a; Martino et al 1992). A further reversible detoxification occurs when 4-hydroxyifosfamide reacts with sulphydryl groups present in proteins or amino acids, forming 4-thioifosfamide. The inactive metabolite alcoifosfamide has been detected only in animals (Struck et al 1983).

2.4.4 Dechloroethylation of ifosfamide

Dechloroethylation leads to the formation of 2- and 3-dechloroethylifosfamide (2-DCEI and 3-DCEI) and chloroacetaldehyde. Ifosfamide and cyclophosphamide metabolism differ quantitatively in this respect (Norpoth 1976a). The mean Cmax and AUC of activated cyclophosphamide are three times higher than those reported for activated ifosfamide, implying that dechloroethylation is the predominant pathway of ifosfamide metabolism, and possibly explaining the need for higher doses of ifosfamide compared to cyclophosphamide. (Brade et al 1985; Goldin 1982; Wagner et al 1981). The dechloroethyl metabolites of ifosfamide were first isolated

in urine in 1976 (Norpoth 1976a; Norpoth et al 1976b). There is a large interindividual variation in the ratio of the side chain/ring dechloroethylation to ring hydroxylation, and it seems that cleavage of the chloroethyl group from the ring nitrogen is preferred to dechloroethylation of the extracyclic nitrogen atom. Dechloroethylation of up to 50% of a dose of ifosfamide to either 2-DCEI or 3-DCEI can occur (Norpoth et al 1975). In physiological conditions 2-DCEI and 3-DCEI are relatively stable. The urinary excretion of 3-DCEI is approximately three times higher than that of 2-DCEI, and 3-DCEI levels in plasma have been found to be higher than those of 2-DCEI, suggesting that dechloroethylation of the nitrogen ring position of ifosfamide is more common than that of the exocyclic nitrogen (Kurowski and Wagner 1993).

Boddy et al (1993) gave paediatric patients ifosfamide infusions of 72 hours duration every three weeks. The concentration of dechloroethylated metabolites in some individuals continued to rise long after the Tmax of ifosfamide. The urinary recovery of dechloroethylated metabolites increased with the course of administration, but corresponding correlations between plasma AUC and treatment course were not observed. A correlation between the AUCs of ifosfamide and its metabolites with the recovery of these compounds in urine was lacking. There was no increase in the excretion of dechloroethyl metabolites during the infusion.

Gilard and co-workers analysed 2-DCEI and 3-DCEI in human urine using [^{31}P]NMR, identifying degradation products of these compounds (Gilard et al 1993). The proportion of dechloroethylated derivatives was higher than that of the metabolites of the activation pathway.

There is a wide variation in the plasma concentrations of chloroacetaldehyde reported in the literature (Cerny and Küpfer 1989; Goren et al 1986; Kaijser et al 1993; Kurowski and Wagner 1993; Kurowski et al 1991). Goren and colleagues detected levels of up to 100 μM in children, following an ifosfamide infusion of 1.6 g m^{-2} day^{-1}, whilst Cerny and Küpfer reported levels of 210 μM. Following a dose of 1.5 g m^{-2} day^{-1} of ifosfamide for five days in adults, Kurowski and Wagner

determined mean chloroacetaldehyde Cmax concentrations of 2.69 μM and 4.85 μM on days 1 and 5 respectively, with corresponding values for 2-DCEI of 8.6 μM and 16.7 μM, and for 3-DCEI of 12.9 μM and 26.5 μM, almost reaching the concentrations of ifosfamide itself.

In vivo, chloroacetaldehyde is rapidly converted to chloroacetate. Between 6% and 22% of chloroacetate administered to mice is eliminated unchanged in the urine (Yllner 1971). The main urinary metabolites of chloroacetate are S-carboxymethyl-L-cysteine and thiodiacetic acid, with glycolic and oxalic acids generated to a lesser extent. As well as transformation to chloroacetic acid, chloroacetaldehyde may also be converted to cysteinyl derivatives and thioacetals. S-carboxymethylcysteine and thiodiacetic acid are excreted in large amounts after an infusion of ifosfamide (Norpoth 1976a).

2.4.5 Factors influencing ifosfamide metabolism

a Enzyme induction

There is evidence that ifosfamide induces its own metabolism (Kurowski and Wagner 1993; Kurowski et al 1991; Lind et al 1989a; Lewis et al 1990; Nelson et al 1976; Piazza et al 1984; Wagner and Drings 1986). Fractionation over a number of days produces a time dependent decrease in the half-life of ifosfamide without an increase in renal clearance. Nelson et al (1976) used a fractionated ifosfamide regimen of 1.6 – 2.4 g m^{-2} daily for 3 days in three patients, with a second course three weeks later, and compared it to a single intravenous bolus dose of 5 g m^{-2} in five patients (Allen and Creaven 1975; Creaven et al 1974). The fraction of ifosfamide metabolised at the lower dose was nearly twice that for the higher dose, and the renal excretion and clearance rates were similar for both regimens. These results suggest that an increase in ifosfamide metabolism occurs when the dose is fractionated. This was confirmed by Piazza et al (1984), who studied ten patients with lung cancer receiving intravenous ifosfamide 1.8 g m^{-2} daily for five

consecutive days, and found nearly a 50% reduction in drug elimination half-life on day 5 compared to day 1.

In a later study, Lind et al (1989a) gave intravenous ifosfamide 1.5 g m^{-2} daily for five days, and noted that the total clearance was increased from a median of 69 ml min^{-1} on day 1 to 123 ml min^{-1} on day 5, although ifosfamide distribution remained constant. Corresponding figures for non-renal clearance were 60 ml min^{-1} and 104 ml min^{-1} for days 1 and 5 respectively. The half-life of ifosfamide decreased from 6.16 hours on day 1 to 3.76 hours on day 5, with respective figures for the AUC of 575 μg.hr ml^{-1} and 284 μg.hr ml^{-1}. This provides further evidence that the pharmacokinetic changes are due to increased metabolism with time, perhaps as a result of increased liver enzyme activity. Similar results were published by Lewis et al (1990), who demonstrated a significant increase of 76% in ifosfamide clearance over a daily administration period of five days. The apparent volume of distribution was unaffected.

As expected, increased metabolite production occurs with fractionation of ifosfamide dose. The autoinduction of ifosfamide metabolism can be detected by the second day of treatment, and subsequently increases (Boddy et al 1993). Lind et al (1990b) studied the excretion of alkylating metabolites in urine following fractionated ifosfamide 1.5 g m^{-2} daily for five days, administered either orally or intravenously. The median total alkylating metabolite excretion, expressed as a percentage of the ifosfamide dose, increased in the intravenous group from 3.45% to 8.13% by day 5, and in the oral group from 15.18% to 29.15% by day 3. Over the five day period there was no increase in urinary ifosfamide excretion. In the study of Lewis and colleagues, there was a significant increase of more than three-fold in the plasma alkylating AUC on day 5 compared to day 1, whilst Boddy and co-workers detected up to a three-fold increase in ifosfamide clearance during three days of continuous infusion, with an increased rate of dechloroethylation (Boddy et al 1993; Lewis et al 1990).

Hartley et al (1994) studied the urinary excretion of ifosfamide and its metabolites during three day infusions. The proportion of ifosfamide (expressed as a proportion of total urinary drug products) in the urine decreased, but those of 2-DCEI, 3-DCEI and CIPA increased, whilst that of IPM was unchanged. The proportion of each metabolite remained constant with increasing ifosfamide doses, and there was no evidence of saturation of metabolic pathways. Small but significant increases in the fraction of drug converted to 2-DCEI and IPM were observed with successive cycles. Ifosfamide, 3-DCEI and CIPA appeared in the urine progressively earlier in successive cycles of treatment. Analysis of metabolite ratios suggested that patients who metabolised more ifosfamide to 2-DCEI also tended to convert more to 3-DCEI, and less to CIPA.

Kurowski and Wagner (1993) reported that the Cmax and AUC values of 4-hydroxyifosfamide and the dechloroethylated metabolites increased approximately two-fold during a five day treatment schedule. The two metabolic routes were induced to the same extent, and the relative contribution of accumulation to the total increase in metabolite AUC on day 5 was negligible. The average Cmin levels of 4-hydroxyifosfamide, chloroacetaldehyde, 3-DCEI and 2-DCEI accounted for only 2.5%, 4.0%, 5.6% and 4.3% respectively, of the subsequent maximum values.

b Stereoisomerism

Ifosfamide exhibits optical isomerism due to the presence of an asymmetrically substituted phosphorus atom. It is used as the racemic mixture, but since oxazaphosphorines require enzymatic activation, the possibility of stereoselective metabolism exists. In mice, both enantiomers of ifosfamide are eliminated at almost equal rates, whilst KIPA is formed stereoselectively from s(-)-ifosfamide. However this does not result in differences in biological activity (Blaschke et al 1986).

c Age and obesity

Age has a minimal effect on ifosfamide pharmacokinetics. There is a positive correlation between the elimination of ifosfamide and age, with an increased β half-life in elderly patients, explained by a higher apparent volume of distribution (Lind et al 1990a). Total plasma clearance, renal clearance and non-renal clearance are not affected, but changes in the autoinduction of ifosfamide metabolism can occur. The β half-life of ifosfamide is shorter in children than in adults, with a higher renal excretion of ifosfamide and dechloroethyl metabolites (Boddy et al 1993; Boos et al 1992; Goren 1991). Lind et al (1989b) found that the elimination half-life of ifosfamide is prolonged in obese patients, probably as a result of an increase in the apparent volume of distribution, rather than decreased clearance. Therefore toxicity may be more severe in obese patients, although the clinical importance of this is uncertain, given the large interindividual variation in ifosfamide metabolism.

d Individual and genetic variation

There is a large variation in ifosfamide metabolism, and reports describing these appeared as early as 1976 (Kurowski and Wagner 1993; Lind et al 1989a; Lind et al 1990b; Norpoth 1976a). Norpoth (1976a) identified the stable alkylating metabolites in the urine of 25 patients after ifosfamide administration, and suggested a variation in metabolism between individuals of up to 70%; poor metabolisers of ifosfamide were identified, in addition to those preferring dechloroethylation or 4-hydroxylation. The variation in CIPA production has been discussed above. However it is not clear whether all the variations observed can be explained by the genetic determinants of the cytochrome P-450 isoenzymes. Philip et al (1988) measured the plasma pharmacokinetic profiles of ifosfamide and NBP alkylating activity in 33 patients who had been phenotyped for debrisoquine oxidation. Three were poor metabolisers, but no correlation was identified between the debrisoquine metabolic ratio and the total plasma clearance of ifosfamide, nor the AUC of the plasma NBP alkylating activity.

e Dose

Variations in dose and dosage interval affect ifosfamide metabolism. A dose dependent saturation of enzymatic activation has not been firmly identified, although some data from a dose intensification study indicate that ifosfamide metabolism may be saturated at doses above 14 g m^{-2} (Wagner 1994). However, at single doses of up to 5 g m^{-2} the half-life is not prolonged, and the decline in plasma ifosfamide concentrations remains a first order process (Cerny et al 1986).

2.5 Excretion

Ifosfamide and its metabolites are excreted by the kidney, although quantities found in urine are subject to large interindividual variation. Allen et al (1976) found that 53.1% of the administered dose of ifosfamide is excreted unchanged in the urine, but lower concentrations have been reported in more recent studies; 2.58% (Lind et al 1990b), 16.6% (Martino et al 1992), 18% (Goren 1991), and 18% (Gilard et al 1993). In children, a urinary excretion of 14% to 34% has been described (Boos et al 1992). These variations may reflect differences in analytical techniques. Lind and co-workers found that the most common alkylating metabolite in urine was dechloroethylifosfamide, at concentrations similar to those reported by Norpoth; the excretion of CIPA was very variable, and the levels of IPM were low (Lind et al 1990b; Norpoth 1976a).

2.5.1 Elimination half-life

The main route of ifosfamide elimination is metabolism, with excretion playing a minor role. There is a large interindividual variability in half-lives reported in the literature, and the dependence of the activation of ifosfamide on the cytochrome P-450 system may be part of the explanation. In most studies of ifosfamide administered as a bolus injection, or as a short intravenous infusion, at doses of 1 g

m^{-2} to 3 g m^{-2}, the terminal elimination plasma half-life is between 30 minutes and nine hours (Cerny et al 1986; Lewis et al 1990; McNeil and Morgan 1981; Nelson et al 1976; Piazza et al 1984). However, Allen et al (1976) recorded a half-life of 15 hours; differences in analytical techniques may be responsible, as ifosfamide and hydrophobic metabolites, such as 2-DCEI and 3-DCEI, are determined together unless adequate separation steps are taken (Wagner et al 1981). As described previously, a significant reduction in the terminal elimination half-life of ifosfamide during fractionated dosage regimens has been identified.

2.5.2 Clearance

Allen et al (1976) described a renal clearance of ifosfamide of 21.3 ml min^{-1}. Following 1.6 g m^{-2} to 2.4 g m^{-2} intravenous ifosfamide daily for three days, Nelson et al (1976) determined a total body clearance of 14.9 ml min^{-1} to 24.2 ml min^{-1}, and a urinary excretion of unchanged ifosfamide of 12.4% to 17.9% of the total dose, but the assay may have been non-specific. These values of total body clearance are lower than those subsequently reported.

Following ifosfamide administration, 500 mg orally or an intravenous infusion of up to 2 g, McNeil and Morgan (1981) calculated a total body clearance of 108 to 148 ml min^{-1}. The urinary excretion of unchanged ifosfamide was 9.4% to 10.8% of the total dose. Lind et al (1989a) gave 1.5 g m^{-2} day^{-1} oral ifosfamide consecutively for five days to ten patients; a total body clearance of 51 ml min^{-1} was obtained on day 1, increasing to 86 ml min^{-1} by day 5. The group of Cerny compared 1 g and 2 g doses of oral and intravenous ifosfamide, reporting total body clearances of 45 to 72 ml min^{-1}, and 61 ml min^{-1} after 5 g oral ifosfamide (Cerny et al 1986). Similarly, Lewis et al (1990) described a total clearance of ifosfamide of 66 ml min^{-1} on day 1. A renal ifosfamide clearance of 0.663 L hr^{-1} m^{-2} in children indicates that this is not an important route of elimination in this age group (Boddy et al 1993).

2.6 Conclusion

Ifosfamide possesses complex pharmacokinetics and metabolism is central to its cytotoxic activity. The competing processes of dechloroethylation and 4-hydroxylation are probably important determinants of activity and toxicity in any given individual. Despite advances in pharmacokinetic understanding, the ultimate fate of up to 70% of an intravenous ifosfamide dose is not known. The formation of metabolites not detected by the currently available assays is a possibility. Other alternative explanations are the conjugation of parent drug or reactive metabolites to mesna, or endogenous compounds such as glutathione, perhaps followed by biliary excretion and faecal elimination. It is known that cyclophosphamide is eliminated in the bile, but only 0.92% to 2.5% of intravenously administered ^{14}C-labelled cyclophosphamide is found in the faeces (Bagley et al 1973; Dooley et al 1982).

Chapter 3

The Central Nervous System Toxicity of Ifosfamide

3.1 Introduction

Urotoxicity was the dose limiting side effect of ifosfamide until the introduction of mesna in 1983, when it was replaced by myelosuppression and neurotoxicity. Encephalopathy of varying degrees occurs in approximately 10% of patients after intravenous ifosfamide, and is almost always reversible, but has occasionally proved fatal. The condition is reported less frequently in children. This chapter reviews the clinical features of ifosfamide induced encephalopathy, associated risk factors, and possible aetiological agents and mechanisms of development.

3.2 Clinical features

3.2.1 Incidence

Initial reports of encephalopathy following ifosfamide and mesna suggested an incidence of approximately 5%, but these effects were poorly characterised (Brühl et al 1976; Scheulen et al 1983; Schnitker et al 1976). In 1982, Morgan and co-workers noted CNS toxicity in 13 of 254 (5.1%) NSCLC patients treated with either ifosfamide 4 g m^{-2} day 1, or 1.2 g m^{-2} days 1 to 5, without mesna (Morgan et al 1982). In a later study, an overall incidence of 11% was found in adults with a variety of tumour types, using a dose of 5 g m^{-2} over 24 hours (Watkin et al 1989). Severe encephalopathy was described in five of 31 women (16%) with cervical or endometrial carcinoma given ifosfamide 5 g m^{-2} and mesna 8.2 g m^{-2}, and seven of 41 patients (17.1%) with advanced carcinoma of the cervix developed encephalopathy following single agent ifosfamide 1.5 g m^{-2} daily for five days (Coleman et al 1986; Meanwell et al 1985).

Both higher and lower incidences have also been reported. In one study of a 24 hour intravenous infusion of 5 g m^{-2} ifosfamide, with a total dose of intravenous mesna of 5 g m^{-2} per cycle, serious CNS toxicity occurred in less than 1% of 103 patients with small cell lung cancer (SCLC) (Cantwell and Harris 1985). Perren et al (1987) observed adverse CNS reactions in three of five patients with advanced lung cancer treated with a six hour infusion of ifosfamide 5 g m^{-2} with mesna 5 g m^{-2}. In another series of patients, CNS toxicity was seen in 5/18 (28%) given ifosfamide, either as a single agent or as part of a combination chemotherapy regimen (Merimsky et al 1991). An incidence of 22% (11/50) was reported in a paediatric population receiving single agent ifosfamide (Pratt et al 1986).

3.2.2 Symptoms

Encephalopathy is usually manifested by somnolence, although other features such as forgetfulness, nightmares, seizures, tonic clonic spasms, and coma may occur. Disorientation, motor unrest, emotional lability, depression, echopraxia, and logorrhoea have also been described (Brühl et al 1976; Miller and Eaton 1992; Rodriguez et al 1976). Both auditory and visual hallucinations have been documented (Heim et al 1981; Watkin et al 1989). The visual hallucinations usually differ from those experienced in psychiatric conditions in that they are often vivid, complex and frightening. DiMaggio et al (1994) described six patients with ifosfamide induced visual and/or auditory hallucinations in the presence of a clear sensorium; all were alert and fully orientated. Interestingly, patients experienced hallucinations only during periods of eye closure.

With severe and progressive encephalopathy, logorrhoea, palilalia, perseveration of writing and speech, and marked disorientation appear after 20 to 50 hours, following which patients may enter a trance, and coma soon follows. Brainstem reflexes are intially preserved, but circulatory collapse secondary to depression of vital centres is eventually fatal.

Seizures, cerebellar signs and cranial nerve dysfunction are more common in children than in adults. Most of the eleven encephalopathic paediatric patients described by Pratt et al (1986) developed drowsiness and somnolence. Mental changes were seen, ranging from irritability and confusion to disorientation and psychosis, and many exhibited personality and behavioural disturbances. Symptoms resolved within three days of stopping ifosfamide. Mild to moderate weakness often developed, and there was one case each of aphasia, ataxia and seizures, with two developing reversible cranial nerve abnormalities. Salloum et al (1987) reported a fatality following ifosfamide induced encephalopathy in a four year old girl. This occurred on the eighth course of treatment, possibly as a result of progressive subclinical renal toxicity during the first seven courses, with impaired clearance of neurotoxic metabolites.

The WHO and CTC gradings for neurotoxicity are shown in tables 3.1 and 3.2.

Grade				
0	**1**	**2**	**3**	**4**
Alert	Transient lethargy	Somnolence < 50% of waking hours	Somnolence > 50% of waking hours	Coma

Table 3.1. WHO grade state of consciousness.

Biochemical disturbances and localising neurological signs are not characteristic, although a low serum bicarbonate, sodium, potassium, and pH have been reported. Signs of water intoxication due to inappropriate arginine vasopressin secretion have also been described (Cantwell et al 1990). Computerised tomographic brain scans have not shown abnormalities, other than pre-existing asymptomatic metastases, which are themselves risk factors. Morphometric studies have revealed no correlation between the severity of the encephalopathy and the width of the ventricles or the sulci

Toxicity	Grade				
	0	1	2	3	4
Neurosensory	none or no change	mild paraesthesia, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paraesthesia	severe objective sensory loss, or paraesthesia that interfere with function	___
Neuromotor	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neurocortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neurocerebellar	none	slight incoordination, dysdiadocokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	cerebellar necrosis
Neuromood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuroheadache	none	mild	moderate or severe, but transient	unrelenting and severe	___
Neuro-constipation	none or no change	mild	moderate	severe	ileus >96 hrs
Neurohearing	none or no change	asymptomatic hearing loss on audiometry only	tinnitus	hearing loss interfering with function, but correctable with hearing aid	deafness, not correctable
Neurovision	none or no change	___	___	symptomatic, subtotal loss of vision	blindness

Table 3.2. Common Toxicity Criteria (CTC) (neurological).

(Merimsky et al 1991), and lumbar punctures and magnetic resonance brain scans have been reported as normal (Danesh et al 1989; Miller and Eaton 1992; Zulian et al 1995). Perren et al (1987) described a patient with a single CNS metastasis, previously treated successfully with radiotherapy, who behaved in an atypical fashion with the development of a mild left hemiplegia and drowsiness 40 hours after ifosfamide treatment; a computerised tomography scan showed a diffuse decrease in density throughout the white matter, with some areas of irregular uptake after contrast.

3.2.3 Timing

The time of onset of encephalopathy is variable; it may occur within two hours of the start of the infusion, or not appear until 28 days, but is most often seen after 20 to 50 hours. The duration of the acute phase can vary between one and twelve days. Encephalopathy most often develops on the first cycle. Watkin et al (1989) described 18 cases of serious encephalopathy during the first, second, third and sixth cycle in eleven, two, four and one patients respectively. One developed encephalopathy during a 24 hour ifosfamide infusion. The mean time to onset was 46 hours from the start of the infusion (range 12 – 146 hours), and the median duration three days (range 1 – 12 days). Five patients took one week or more to recover, and there was incomplete recovery in two patients who died on days 22 and 29, with persistent clinical evidence of encephalopathy. Heim et al (1981) performed a psychiatric assessment, including tests for long and short term memory, before and after treatment with ifosfamide in ten nephrectomised patients with advanced renal carcinoma. Seven patients developed psychological disturbances commencing two hours after the infusion. Emotional instability and impairment of short term memory persisted for up to ten weeks in five and four patients respectively. The previous nephrectomies may have contributed to this high incidence.

3.2.4 Management

Following the development of encephalopathy, ifosfamide should be stopped and any electrolyte abnormalities corrected. Sedative drugs can compound the problem and should be avoided, although the use of high potency neuroleptics such as haloperidol, titrated to sedation if necessary, has been recommended in very disturbed patients (DiMaggio et al 1994; Manegold et al 1992). The presence of intracranial metastases should be considered. Patients may not experience CNS toxicity if re-challenged, despite the persistence of risk factors. If further administration is attempted, a prolonged continuous intravenous infusion of ifosfamide with simultaneous equidose mesna, the correction of hypoalbuminaemia, and bicarbonate supplements, may prevent CNS toxicity. Piracetam and phenytoin have been assessed as a prophylaxis (Cerny and Küpfer 1992). More recently, methylene blue has become established in the treatment and prophylaxis of ifosfamide induced encephalopathy, at a dose of 50 mg three times daily (Küpfer et al 1994). Haemodialysis improved ifosfamide induced irritability, tremor and ataxia in a 20 month old child (Carlson et al 1998).

3.3 The electroencephalogram in ifosfamide encephalopathy

Abnormalities in the electroencephalogram (EEG), consistent with a generalised disturbance, precede clinical symptoms by 12 to 24 hours (Meanwell et al 1985). Clinical symptoms and EEG abnormalities are highly correlated, but early EEG disturbances develop in 56% of patients receiving a 24 hour infusion of ifosfamide without clinical toxicity. During the first 18 hours of treatment, asymptomatic reduced dominant EEG frequency may occur. This is followed by a further lowering of EEG background frequency, with high amplitude paroxysmal delta activity. After 20 to 50 hours, low amplitude 1 Hz delta activity, some theta activity and triphasic waves with an anterior emphasis become evident. A slow reversal of the EEG abnormalities from about 40 hours leads to clinical recovery after a further 36 hours. Subsequent progression of clinical and EEG abnormalities is associated with a worse

prognosis. After 50 hours there may be a further lowering of EEG amplitude and frequency (1.5 Hz – 2 Hz), disappearance of most high frequency components, and the absence of triphasic components. A grading system for ifosfamide induced EEG abnormalities has been described (Table 3.3) (Meanwell et al 1986a).

Grade	EEG recording	Clinical observation
0	Normal	Alert
1	A drop in alpha frequency compared with pretreatment EEG, with or without small amounts of paroxysmal theta activity	Transient lethargy
2	Predominant theta activity, with or without intermittent delta activity	Somnolence < 50% of the time and/or mild/moderate disorientation
3	Predominant delta activity with or without sharp (complex) wave forms	Somnolence > 50% of the time and/or severe disorientation, echolalia, perseveration of writing, palilalia, logorrhoea, hallucinations or delusions
4	Continuous delta activity, complex wave forms and triphasic waves	Coma

Table 3.3. The Meanwell grading of EEG abnormalities.

Other authors have reported generalised or focal abnormalities, or abnormal waves (Coleman et al 1986; Heim et al 1981; Zulian et al 1995). Pratt et al (1986) performed EEGs in 23 paediatric patients who received 51 courses of ifosfamide. Intravenous ifosfamide, 1.6 g m^{-2} over 15 minutes, was given daily for five days. Abnormalities were seen during five courses in four patients with neurotoxicity. Interestingly, during 42 courses in 19 asymptomatic patients, EEGs became abnormal in 14 courses. In all patients, slowing of the baseline rhythm was followed by increased delta activity. One patient, who showed spike activity on the last day of course 1, had a seizure on day 2 of course 2. The EEG was normal before the seizure,

but delta activity subsequently appeared. Danesh et al (1989) described high amplitude, frontally dominant, triphasic waves with a reduction of alpha frequency to 6 Hz, whilst Gieron and colleagues found EEG changes to parallel the clinical course in two children (Gieron et al 1988). There is no data suggesting that pre-treatment EEGs predict the development of clinical encephalopathy.

3.4 Risk factors

Numerous risk factors for the development of encephalopathy have been described. The most important are impaired renal function, low serum albumin, and the presence of pelvic disease or brain metastases. A reduced serum albumin may be an indicator of hepatic impairment, and therefore altered ifosfamide metabolism. Furthermore, any protective protein binding of toxic metabolites will be diminished. Other factors include a pre-treatment bicarbonate of less than 15 mM L^{-1}, a poor performance status, an advanced age, prior brain irradiation, or a history of previous CNS disorders or alcoholism (Manegold et al 1992). A rapid rate of ifosfamide infusion and previous cisplatin therapy have also been implicated (Goren et al 1987; Perren et al 1987).

3.4.1 The Meanwell nomogram

Using discriminant analysis, Meanwell et al (1986a) identified low serum albumin, high serum creatinine and the presence of pelvic disease as variables predisposing to encephalopathy. The study was based on 77 patients with advanced carcinoma of the cervix, treated with a bolus dose of 1.5 g m^{-2} mesna, followed by 5 g m^{-2} ifosfamide and 5 g m^{-2} mesna over 24 hours, then further mesna 3.0 g m^{-2} to 3.2 g m^{-2} over 12 hours. Seven patients (9%) developed WHO grade 3 or 4 CNS toxicity. A nomogram was constructed (figure 3.1). The probability of not developing grade 3 or 4 encephalopathy falls on the intersection of a straight line joining the serum albumin and serum creatinine concentrations on the appropriate pelvic disease scale.

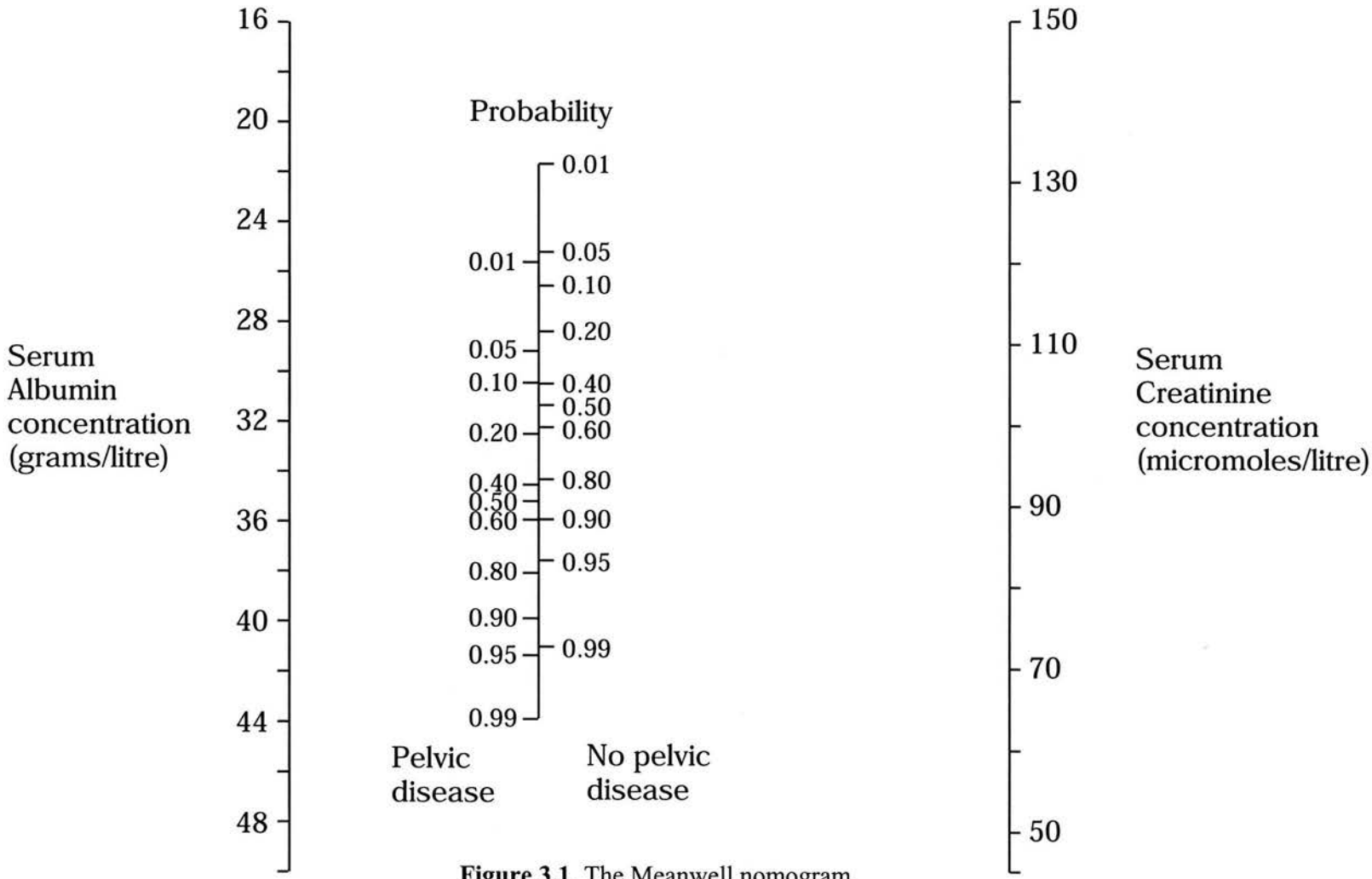


Figure 3.1. The Meanwell nomogram.

The same group performed a prospective study of the nomogram in 45 patients, 22 of whom had cervical carcinoma (Meanwell et al 1986b). Thirty-eight patients had a greater than 0.20 computed probability of remaining free from grade 3 or 4 CNS toxicity, and none developed clinical signs or symptoms of such toxicity (predictive value of a negative test result 100%). Four of seven patients with a probability of less than 0.20 developed grade 3 toxicity (predictive value of a positive test result 57%; 95% CI 20% – 84%). It was recommended that ifosfamide is not administered to patients with less than a 0.20 probability of remaining free from grade 3 or 4 CNS toxicity.

However the universal applicability of the nomogram has been questioned. Two patients with encephalopathy described by Perren et al (1987) had calculated probabilities of freedom from encephalopathy of 0.30 and 0.39. They postulated that their six hour ifosfamide infusion, rather than the 24 hour ifosfamide infusion used by the group of Meanwell, may have resulted in higher serum and CSF levels of the unchanged drug and metabolites, with encephalopathy developing despite lower degrees of renal and hepatic impairment. It was suggested that if the nomogram is used with schedules other than the 24 hour infusion from which it was derived, then the lowest computed probability score should be determined for each schedule. Similarly, Watkin et al (1989) assessed 18 cases of ifosfamide induced encephalopathy following ifosfamide 5 g m^{-2} over 24 hours, either as a single agent or in combination. The Meanwell nomogram was used retrospectively to determine the sensitivity of the test. Only three patients had a probability of less than 0.20 of remaining free of grade 3 or 4 encephalopathy. The sensitivity was 18% (95% CI 0% – 36%). In another report, three of 27 patients with SCLC given ifosfamide developed severe neurotoxicity despite Meanwell probabilities of remaining free from encephalopathy of 0.9 or more (McCallum 1987). The efficacy and accuracy of the nomogram has also been criticised on the basis of the variation in serum albumin with the method of laboratory measurement and posture (Hill and Harrop 1986).

3.4.2 Dose and schedule of ifosfamide

The development of neurotoxicity is dependent on the total ifosfamide dose, dose limiting toxicity occurring at approximately 18 g m^{-2}, and the schedule of administration, with less toxicity when ifosfamide is infused continuously over several days, rather than in daily fractions (Cerny et al 1990a; Elias et al 1990). Serious encephalopathy has been seen following rapid infusion over six hours (Perren et al 1987). A five day schedule may be associated with less severe neurological toxicity than a one day schedule (Coleman et al 1986). Curtin and co-workers treated 18 patients with a 24 hour infusion of ifosfamide 2.5 g m^{-2} to 5 g m^{-2}; six developed WHO grade 3 neurotoxicity, which was not seen in five patients receiving a five day infusion at a daily dose of 1.2 g m^{-2} to 1.5 g m^{-2} (Curtin et al 1991). A dose response relationship has also been identified following oral ifosfamide, when an increase in the daily dose results in more severe neurotoxicity. The total cycle dose is less important than the total daily dose (Manegold et al 1992).

3.4.3 Oral administration

Neurotoxicity occurs more frequently when ifosfamide is given by the oral route, with an incidence of 50%. Wagner and Drings (1986) found four of six patients experienced neurotoxicity after 2 g m^{-2} of oral ifosfamide. Cerny et al (1989b) used 2 g ifosfamide daily, days 1 to 3, with oral etoposide, in SCLC, and one third of patients developed mild CNS toxicity. In a study of oral and intravenous ifosfamide in NSCLC, ifosfamide 1.5 g m^{-2} daily was administered for five days by both routes to groups of ten patients (Lind et al 1989a). Two patients in the oral arm became comatose for 24 to 48 hours, but recovered completely, with no neurological sequelae, whilst three suffered WHO grade 3 neurotoxicity. Mild degrees of grade 2 encephalopathy were seen in the intravenous arm. Manegold et al (1992) studied four different schedules of oral ifosfamide (1 g daily for five days; 750 mg, 1 g or 1.25 g daily for 14 days; [schedules 1 to 4 respectively]), using the Meanwell grading (table 3.3). Grade 1 or 2 CNS toxicity was noted in 2/15 (13%) of patients on schedule 1,

8/19 (42%) on schedule 2, 8/23 (35%) on schedule 3, and 4/7 (57%) on schedule 4. Grade 3 toxicity was not seen.

3.4.4 The influence of disease

It is not clear whether ifosfamide encephalopathy is a disease specific phenomenon, although Meanwell and colleagues felt that it is more likely to occur in patients with bulky, responsive, cervical and endometrial cancers (Meanwell et al 1985). It seems improbable that encephalopathy is associated with response to chemotherapy, and cases have been described in patients with ifosfamide resistant tumours. The products of tumour lysis entering the CNS has been postulated as one mechanism, but Merimsky et al (1991) found no relationship between tumour response, including that of Non-Hodgkins lymphoma, and the development of encephalopathy, suggesting that tumour lysis is not a factor.

3.4.5 Plasma potassium concentrations

In one series of encephalopathic patients, plasma potassium concentrations were available in seventeen patients, and fell from a mean of 4.12 mM before chemotherapy to 2.94 mM at the onset of encephalopathy, and to a minimum of 2.88 mM (Watkin et al 1989). In 14 patients the plasma potassium nadir was at the time of onset of the encephalopathy, and in three the potassium fell subsequently. In two, hypokalaemia was attributable to prolonged vomiting. Hypokalaemia is an unlikely pathogenic factor in the encephalopathy, since there was no relationship between their durations, and it may be a manifestation of tubular damage. It was also postulated that hypokalaemia is caused by the encephalopathy, and an example of stress hypokalaemia, which is thought to be an adrenergic effect.

3.5 Aetiology of ifosfamide induced encephalopathy

The reversibility of ifosfamide induced encephalopathy, the EEG findings of a generalised cortical disturbance, and an increased incidence with higher doses, implicate a metabolic encephalopathy. However the chemical entity responsible has not yet been firmly identified. It is likely to be a metabolite of ifosfamide, and the evidence for this possibility is reviewed in this section.

3.5.1 Ifosfamide

Radiolabelled ifosfamide can be detected within the CSF two hours after an ifosfamide infusion. Concentrations after two to three hours are between 23% and 49% of the plasma level (Creaven et al 1974), but only very low CSF concentrations of ifosfamide metabolites are found, as measured by NPB alkylating activity. Ninane et al (1989) measured CSF alkylating activity in four children after one hour ifosfamide infusions, and this was always lower than the plasma concentration. The mean CSF to plasma concentration ratio during the first 12 hours was 0.53. These observations led to the hypothesis that encephalopathy may be a consequence of a direct toxic effect of ifosfamide on the CNS. However, the studies were unable to assess the contribution of ifosfamide metabolites, as the NBP reaction is an insensitive test for these compounds. Ifosfamide concentrations in paediatric CSF have been reported to be almost as high as in plasma, with CSF to plasma ratios of 0.59 to 2 (Kaijser et al 1997; Yule et al 1997).

In patients with ifosfamide induced encephalopathy, the clearance, half-life, and volume of distribution of ifosfamide are not significantly different to those obtained in patients without this complication, suggesting that a metabolite is responsible.

3.5.2 Mesna

Mesna can produce somnolence and fatigue, but little is known of its penetration into the CSF. The compound binds several metals to albumin, including copper, iron and lead. It has been postulated that neurotoxicity results from large unusual fluxes of intra and extra cellular iron or copper ions, disturbing adrenergic and serotonergic central neurotransmitter systems (Meanwell et al 1985). However it is very unlikely that mesna is responsible for CNS toxicity, as ifosfamide encephalopathy was seen prior to its introduction. Mesna may even protect against CNS toxicity, as it neutralises 4-hydroxyifosfamide, chloroacetaldehyde, acrolein and IPM. The lethal effects of small amounts of chloroacetaldehyde (5 ppm) in a fish model of neurotoxicity can be prevented by adding mesna to the water (Cerny and Küpfer 1992).

3.5.3 Chloroacetaldehyde and dechloroethylation

Chloroacetaldehyde may be a factor in the development of neurotoxicity. It is neurotoxic in rats (Lawrence et al 1972), and structurally related to chloral hydrate (trichloroacetaldehyde), which in overdose produces symptoms and signs similar to ifosfamide induced encephalopathy. Neurotoxicity has not been seen following cyclophosphamide administration, even after the high doses associated with peripheral stem cell collection and transplantation; early reports suggested that lower quantities of chloroacetaldehyde are probably generated following cyclophosphamide metabolism (Bagley et al 1973; Colvin 1982; Norpoth 1976a). Goren et al (1986) pointed out that as 50% of ifosfamide can be dechloroethylated, potentially leading to ten-fold greater quantities of chloroacetaldehyde, and since the dose of ifosfamide is usually ten times that of cyclophosphamide, the combined difference in side chain dechloroethylation and dosage suggests that ifosfamide may yield one hundred times more chloroacetaldehyde than cyclophosphamide.

A high reactivity and short half-life of three to five minutes make chloroacetaldehyde notoriously difficult to quantify in biofluids. Goren et al (1986) found mean blood chloroacetaldehyde levels of 88 μM at six hours and 109 μM at 24 hours in two patients with neurotoxicity, compared with respective concentrations of 45 μM and 22 μM in four patients without such toxicity. They postulated that the persistently high levels and temporal relationship to the development of neurotoxicity implicated chloroacetaldehyde as the cause. Cerny et al (1990a) were unable to confirm the chloroacetaldehyde threshold of 80 μM to 100 μM, above which neurotoxicity is likely to develop, as suggested by the group of Goren; it was not seen at levels of up to 210 μM.

Kurowski et al (1991) performed a cross over study of intravenous and oral ifosfamide, and found higher plasma concentrations of chloroacetaldehyde with the oral route. However in a later intravenous study, concentrations on the last day of a five day ifosfamide schedule of 1.5 g m^{-2} day^{-1} were just as high as those following the same dose given on one occasion orally in the initial study, and no neurotoxicity was seen.

Dechloroethylifosfamide has been used as an indicator of chloroacetaldehyde formation. In one study a planned five day course of oral ifosfamide often had to terminated following neurotoxicity, usually on day 3 (Lind et al 1990b). Oral administration resulted in a higher recovery of dechloroethylifosfamide, IPM and CIPA from urine, but there was no relationship between any individual metabolite and the development of neurotoxicity. A possible explanation is a poor correlation between urinary and plasma concentrations. In a study of ten day continuous infusions of ifosfamide in 23 patients, one developed neurotoxicity after six days, associated with high 2-DCEI and 3-DCEI plasma levels (greater than 40 μg ml^{-1} compared with less than 14 μg ml^{-1} for those without encephalopathy) (Kaijser et al 1996). The penetration of 2-DCEI and 3-DCEI into CSF is low in children, levels either being undetectable or reaching CSF to plasma ratios of less than 0.5 (Kaijser et al 1997; Yule et al 1997). The stereochemistry of dechloroethylation may also be

important, and a relationship between CNS toxicity and increased urinary excretion of (R)-3-DCEI has been postulated (Wainer et al 1994).

Although the bioavailability of oral ifosfamide is almost 100% (Cerny et al 1986; Wagner and Drings 1986), a small but significant first pass effect would be compatible with a very toxic metabolite, such as chloroacetaldehyde, preferentially being produced following oral dosing. The oral route generates higher biliary chloroacetaldehyde levels in rats compared to the intravenous route (Cerny and Küpfer 1992). Following oral cyclophosphamide higher levels of serum alkylating activity have been found compared to intravenous administration, despite an oral bioavailability of almost 100%, suggesting the production of a different profile of metabolites (Juma et al 1979). It is still not certain whether the differences seen after oral administration are the result of a hepatic effect or even earlier presystemic metabolism.

Glutathione, a tripeptide thiol, may influence clinical response to ifosfamide by deactivating alkylating species through conjugation of nucleophilic groups with its sulphydryl groups. Glutathione has been incorporated into a possible mechanism for the neurotoxicity after oral ifosfamide (Lind et al 1989c). It has been postulated that higher hepatic levels of ifosfamide metabolites after oral administration results in a more pronounced fall in hepatic glutathione than after intravenous administration, when ifosfamide is distributed throughout the total body water. The liver may then be unable to detoxify chloroacetaldehyde after oral administration, with elevated blood levels of this compound. Furthermore, ifosfamide induced encephalopathy is associated with lower plasma and whole blood levels of glutathione (Mulders et al 1995).

An interesting development in unravelling possible metabolic causes of the neurotoxic syndrome is the use of methylene blue. This compound has been employed for many years in the treatment of cyanide poisoning by virtue of its capacity to act as an electron acceptor. Urinary sarcosine and glutaric acid levels are elevated in patients with ifosfamide induced encephalopathy, implying an

impairment of electron transport within the CNS mitochondria, and the neurotoxicity has been reversed successfully with methylene blue (Küpfer et al 1994). This important pharmacodynamic interaction with methylene blue indicates the site of action of the causative agent, but does not provide absolute identification. It further supports the possibility that chlorinated metabolites are responsible for the encephalopathy, as chloroacetaldehyde is known to poison mitochondria. However, using ^{1}H NMR analysis of urine from five encephalopathic patients, Foxall and co-workers were unable to detect glutaric acid, and in two patients who received methylene blue neurological symptoms were not improved (Foxall et al 1997).

3.5.4 Aziridino ifosfamide

Aziridino ifosfamide, a possible metabolite of ifosfamide, has been proposed as a causative agent (Cerny and Küpfer 1992). The only other alkylating agent capable of generating neurotoxicity similar to that seen after ifosfamide is thio-TEPA, also an aziridine, at doses exceeding 900 mg m^{-2} (Wolff et al 1990).

3.5.5 4-hydroxyifosfamide

Serum 4-hydroxyifosfamide levels after oral ifosfamide are twice those following intravenous administration (Kurowski et al 1991). In three Rhesus monkeys given 1 g m^{-2} of ifosfamide, a 4-hydroxyifosfamide CSF to blood ratio of 0.13 was found, higher than the ratio for NPB alkylating activity (Arndt et al 1988). One animal became neurotoxic, but the levels of 4-hydroxyifosfamide in the CSF were no higher than in the unaffected monkeys. 4-hydroxyifosfamide and IPM enter the CSF easily in children, with CSF to plasma ratios of 0.83 to 0.92, and 3.2, respectively (Kaijser et al 1997; Yule et al 1997).

3.6 Conclusion

Neurotoxicity following ifosfamide administration remains a significant problem, particularly with the oral route. An increased awareness of predisposing factors has permitted the safer selection of patients, and the recent introduction of methylene blue has allowed prophylactic measures to be taken. The phenomenon remains to be adequately explained, and although chloroacetaldehyde may be contributory, other as yet unidentified metabolites, or processes, could also be important.

Chapter 4

The Erythrocyte Compartment

4.1 Introduction

Quantitatively, the three most important transport fractions of blood are plasma water, plasma proteins and cells, mainly erythrocytes. In the laboratory, this three compartmental system, equilibrating in the circulation in the dark and at 37°C, is reduced for practical purposes to the two compartmental system of plasma, and analysed at room temperature as if it had only one compartment. The information present in the three separate fractions of whole blood is therefore combined into one value. In the case of a compound which is bound to plasma proteins, this composite value is misleading as a change in the free fraction present in plasma water can be hidden by the large quantity present on proteins. Binding to erythrocytes also occurs, but even less consideration has been given to the role of these cells in the transport of compounds other than oxygen.

There are three reasons for this neglect of the erythrocyte and subsequent lack of knowledge. Firstly, conceptual difficulties exist, as illustrated by the following statement: "The erythrocyte has a cell wall, so it really has its own compartment. Distribution in body fluids does not take effect versus the fraction free in whole blood, but versus the free concentration in plasma water. From the pharmacokinetic point of view, I would say that the red blood cells have the lowest priority" as discussed by a pioneer on erythrocyte studies, M. Ehrnebo (Ehrnebo 1986). As a result, the transport role of erythrocytes is almost invariably investigated using washed erythrocytes, as if these cells were indeed separate compartments, and regardless of the fact that washed erythrocytes do not exist in the circulation (e.g. Jourdil et al 1993; Zingales 1973). Secondly, the transport function of red cells is influenced by physiological factors as the role of erythrocytes will often become more

apparent when protein binding is saturated, and thirdly, bioanalytical limitations restrict the investigation of the transport role of erythrocytes.

Traditionally, plasma proteins have been viewed as the carriers of drugs, in equilibrium with plasma water, whilst the erythrocytes have been regarded as a transport system for blood gases, which bind to proteins within the red cell. However, the erythrocyte can possess a more extensive transport function. Compounds with a strong affinity for intracellular proteins may be sequestrated within the erythrocyte. Such binding does not necessarily imply that compounds are unavailable to the tissues, but with the exception of oxygen the transport mechanisms have been poorly defined. For most compounds showing an association with erythrocytes, it is not known whether they primarily bind to the outside of the erythrocyte, penetrate the cell membrane, or if both mechanisms are operative. Despite these uncertainties, erythrocytes may be of great importance in transferring such compounds to the tissues.

4.2 Indications for the analysis of the red cell compartment

In many clinical situations the analysis of plasma alone is adequate. As the relationship between erythrocyte cargo and plasma water concentration is often stable, if desired the quantification of the amount associated with the erythrocyte is a satisfactory alternative to the measurement of concentrations in plasma water. One can also envisage some circumstances in which a more elaborate analysis of the transport system in whole blood is indicated.

A Studies where the erythrocyte itself is the subject of investigation. Red cells have been used as drug carriers, particularly of cytotoxic agents (Kravtzoff et al 1990; Tonetti et al 1991), and for certain compounds they can act as a bioreactor (Cossum 1988; Nagasawa et al 1992; Schmiegelow and Bruunshuus 1990). In the treatment of malaria, the penetration of antimalarial drugs into the erythrocyte is obviously a critical step (Dua et al 1993; Tajerzadeh and Cutler 1993), and in nutritional studies

erythrocyte vitamin content is frequently measured (Anderson et al 1989; Fanelli-Kuczmarski et al 1990). Events in the erythrocyte can often reflect those in other tissues. For example, the red cell folate level is a widely used indicator of the folate status of an individual. More recently, erythrocyte glutathione levels have been proposed as a predictor of tissue intracellular glutathione concentration (Hercbergs et al 1992), and as a biological marker for the severity of cystic fibrosis (Mangione et al 1994), whilst in children receiving maintenance 6-mercaptopurine for acute lymphoblastic leukaemia, the level of erythrocyte 6-thioguanine nucleotides (ETGN) has been found to be important. One study found a correlation between mean ETGN and the degree of myelosuppression (Schmiegelow and Bruunshuus 1990), and in another report a multivariate analysis showed that ETGN concentration was an independent prognostic variable (Lilleyman and Lennard 1994).

B Studies involving the distribution and mutual displacement of body constituents, foodstuffs and xenobiotics through various blood compartments (Colburn and Gibaldi 1978; Melander et al 1979). The mutual interaction of valproate and phenytoin is one such example (Meijer 1991). Sometimes the concentration in the erythrocyte compartment is greater than that in the plasma, as in the case of desethylamiodarone, a metabolite of amiodarone (Maling et al 1989). A physiological model of hydrocortisone transport has been described, in which the erythrocyte is central to the delivery of this compound to tissues, on account of the faster rate of dissociation of erythrocyte bound hydrocortisone compared to that bound to cortisol binding globulin (Hiramatsu and Nisula 1988). One would also expect that in certain abnormal conditions (e.g. hyperbilirubinaemia, uraemia), when large quantities of pathophysiological products bind to plasma proteins, the secondary transport system will be loaded more heavily (Mabuchi and Nakahashi 1988; Shirkey et al 1985).

Therefore, in the case of certain compounds and in some pathophysiological conditions, it is worthwhile investigating the relationship between the concentration on plasma proteins, the steady state load on erythrocytes and the physiological consequences; some desired or undesired effects may be more closely related to erythrocyte load than to the concentration on (nearly saturated) plasma proteins. For

example, in neonates with erythroblastosis foetalis, an unconjugated plasma bilirubin concentration of 300 μM or more leads to the uptake of bilirubin into the CNS, which may result in kernicterus. This pivotal plasma concentration may also be accompanied by a manifest loading of erythrocytes.

4.3 The analysis of the erythrocyte compartment

4.3.1 Methods used in the literature

After collecting a blood sample it is easy to obtain plasma by centrifugation, but the separation of plasma water from plasma is more laborious and not routinely performed in clinical laboratories. The degree of plasma protein binding of a substance is calculated by subtracting the amount present in the plasma water, called the free fraction, from that present in total plasma.

The red cell fraction presents the most obstacles when analysing the three main blood fractions, as the residual cell sediment formed following the centrifugation of blood is difficult to analyse quantitatively using normal volumetric procedures. One approach to this problem is to measure the volume of the cell sediment in a calibrated centrifuge tube. However it is then necessary to wash the sediment with saline or buffer several times before analysis, in order to remove the plasma trapped between the cells; this has the disadvantage of disturbing the normal equilibrium between red cells and plasma water (Ehrnebo et al 1974; Garcia-Alvarez et al 1993; Jourdil et al 1993; Reinhart 1993; Reinhart and Rohner 1990; Wanner et al 1990; Zingales 1973). The effects of such a washing procedure on the results subsequently obtained are rarely mentioned (Bower 1982; Reinhart 1993; Rustad 1964), and the washing itself is never analysed. A second approach, in which the erythrocytes remain in their natural environment, but where the analysis is indirect, is also common in the literature. The amount associated with red cells is calculated by subtracting the amount present in plasma, corrected for the haematocrit, from that present in whole blood (not a medium in which concentrations are routinely measured) (Bower 1982;

Ehrnebo et al 1974; Kravtzoff et al 1990; Maulard et al 1990; Tamura et al 1987). As the quantity associated with red cells is often small compared to the plasma concentration, two large numbers are used to determine a small one. This is an inaccurate method, and occasionally even negative values are obtained when the erythrocyte associated amount is calculated. These problems are frequently not discussed in the literature, and how the recorded erythrocyte concentrations are obtained is often not clear (Anderson et al 1989; Fanelli-Kuczmarski et al 1990; Javaid et al 1985).

4.3.2 Direct analysis of the erythrocyte compartment

The use of appropriate methods is of vital importance for demonstrating the transport role of erythrocytes. The essence is to measure the compounds present in or on unwashed erythrocytes directly. Three studies in which such an approach was used are reviewed (Driessen et al 1989a; 1989b; 1989c; 1990). They concern the compounds valproate, phenytoin, and hydrocortisone. Both in vitro and in vivo work was described.

The method used to process the erythrocytes in these studies involved harvesting blood into haematocrit capillaries and separating erythrocyte sediments by centrifugation at 10,000 g. The capillaries were then cut with a glass knife, and both the length of the cut fragment and its weight determined. Knowing the weight per unit length of capillary tubing, the mass of the cells was obtained. The cell mass was extracted and its drug content analysed. From these measurements a known concentration in w/v of cell mass was derived. A correction for the difference in density between red cells and water was not made. During sample pretreatment, care was taken to maintain the same equilibria as present in whole blood or an erythrocyte suspension in plasma water. Therefore after sampling, the haematocrit capillaries were centrifuged immediately and the sediment separated from plasma or plasma water. The concentration of drug in the red cell sediment was then adjusted for the residual plasma trapped between the cells. This was determined using ^{14}C-inulin as an

extracellular marker and appeared to be 2.0%. During sample pretreatment this trapped plasma may be important to maintain the equilibrium that exists in the circulation. The quantity of drug bound to plasma proteins was calculated by subtracting the quantity in plasma water from that in plasma. In vitro, it was confirmed that the total drug recovery from all the fractions was equal to the amount added to blood, plasma or the mixture of red cells and plasma water.

Compound loss from plasma proteins and erythrocytes in spiked whole blood in vitro, following dilution with blank autologous plasma water was investigated. This reproduced the conditions in capillaries with a discontinuous endothelium, simulating the contact of blood with blank intercellular tissue water following the initial exposure of an individual to a compound. Valproate, phenytoin and hydrocortisone were released relatively easily from the erythrocyte in comparison to plasma proteins, and this finding is supported by other data in the literature for hydrocortisone (Hiramatsu and Nisula 1988). The plasma proteins were saturable with all three compounds, but erythrocytes could not be saturated, not even in high and toxic concentrations. For phenytoin and hydrocortisone the concentrations on the erythrocytes and in plasma water increased proportionally. As varying concentrations of phenytoin and hydrocortisone do not alter the red cell to plasma water ratio, the erythrocyte concentration reflected the concentration in plasma water.

In vivo work showed that the rate of change of valproate concentration is greater on erythrocytes than on plasma proteins, and the same phenomenon occurs when phenytoin is infused linearly over 30 minutes (Driessen et al 1989a, 1989b). A more rapid decline of the red cell fraction is observed following a bolus injection of hydrocortisone (Driessen et al 1990). The elimination half-life from each compartment of whole blood is different; loss from erythrocytes is faster than loss from plasma proteins, and it appears that for these three compounds, those molecules associated with erythrocytes, rather than those bound to plasma proteins, are more likely to exchange with plasma water.

Differences in the erythrocyte and plasma half-lives of other compounds are reported in the literature. For example, in a pharmacokinetic study of a new sulphonamide, highly bound to erythrocytes, the drug was found to have a longer red cell half-life compared to its plasma half-life (Ito et al 1982), whereas the half-life of diazepam on erythrocytes is shorter than its plasma half-life (Hariton et al 1985).

In summary, it seems that for certain compounds, erythrocytes form a secondary transport system in whole blood which, with plasma water, becomes more significant as the non-protein bound drug concentration increases. Data from dilution experiments indicate that erythrocyte bound valproate, phenytoin or hydrocortisone are more readily available to blank plasma water than the protein bound fraction. In vivo observations, using the same compounds, support the hypothesis that erythrocytes act as a secondary transport system, more reluctant to load and faster to discharge than plasma proteins. Although the amount bound to the erythrocytes is less than that bound to plasma proteins, the faster elimination from erythrocytes, coupled with the high number of these cells in the circulation (45% v/v) implies that erythrocytes are important carriers of such compounds. In the case of phenytoin, calculations showed that between one and five hours after administration, approximately 50% of the drug leaving the blood originated from erythrocytes (Driessen et al 1989b).

4.4 Conclusion

Traditionally, the erythrocyte compartment has been neglected in pharmacokinetic studies, and bioanalytical obstacles are a major reason. However, when a direct determination of valproate, phenytoin and hydrocortisone is performed on unwashed erythrocytes, it can be seen that the red cells are a transport system with a high capacity and low affinity compared to plasma proteins. Thus erythrocytes are loaded last and unloaded first. Contrary to previous concepts, it appears that the erythrocyte compartment is a readily exchanging blood fraction, with a transfer function at least as important as the plasma water and protein fractions.

This capability of erythrocytes to carry substances other than blood gases has not been fully appreciated or explored. The plasma concentration of a substance is usually regarded as the optimum reflection of that in tissue cells, but it is likely that the concentration on erythrocytes is at least equally representative.

PART II MATERIALS AND METHODS

Chapter 5

The Analysis of Ifosfamide and its Metabolites in Biofluids

5.1 Introduction

Ifosfamide is stable in vitro and relatively easy to quantify in biofluids. Radiolabelling with ^{3}H and ^{14}C, gas chromatography (GC) using nitrogen flame ionisation detection or electron capture detection, and fluorometric techniques have been employed (Holdiness and Morgan 1983; Kaijser et al 1991b; Kurowski and Wagner 1993; Wagner and Drings 1986; Wagner et al 1981). High-performance liquid chromatography (HPLC) and ^{31}P nuclear magnetic resonance spectrometry have also been developed (Burton and James 1988; Gilard et al 1993; Margison et al 1986; Martino et al 1992).

The first analytical methods for ifosfamide and its metabolites measured only ifosfamide and "total alkylating activity", based on the NBP reaction (Friedman and Boger 1961). In this reaction IPM, CIPA and DCEI have alkylating activity, as does ifosfamide at high concentrations. However it is not certain whether this translates into alkylating activity in vivo. Initial techniques used GC to measure ifosfamide, and total alkylating activity was measured by spectroscopic analysis of the blue colour produced after reaction with NBP. Improved methods used HPLC, but were still unable to separate and measure individual metabolites. The NBP reaction is non-specific, and only an approximation of total alkylating activity is obtained. Despite similar ifosfamide doses and amounts of alkylating activity, the metabolic profiles of ifosfamide metabolites can be quite different, and the total alkylating activity of all ifosfamide metabolites is higher than activated ifosfamide from which IPM is derived. Some pharmacokinetic studies have utilised the liberation of acrolein from the activated metabolites. 7-hydroxyquinolone, formed by reaction with 3-aminophenol, can be detected fluorometrically (Voelcker et al 1979).

With the pairing of analytical techniques such as thin layer chromatography (TLC) with densitometry, and GC with mass spectrometry (GC–MS) (Lambrechts et al 1991), the study of separate metabolites has become possible. A TLC/densitometry method has been described, enabling the separation and analysis of ifosfamide, CIPA, KIPA, IPM, and the dechloroethyl metabolites, in plasma and urine (Boddy and Idle 1992b). Following the separation of metabolites on TLC plates, the plates are treated with NBP and the site of the metabolites identified by the blue colour. Metabolite concentration is then determined by densitometry.

5.2 Gas chromatography – mass spectrometry

The mass spectrometer can be used to establish the molecular weight and structure of organic compounds. The sample is volatised within the spectrometer; the gas-phase ions so formed are separated according to their mass/charge (m/z) ratios, and are then usually detected electrically. The ion-currents corresponding to the different species are amplified and stored on a computer. The peak intensities are plotted as ordinates, in arbitrary units, or normalised with respect to the most important peak (or some other selected peak), which is assigned a value of 100.

5.2.1 Ionisation and fragmentation

The method of ionisation used is dependent on the physical state of the sample, and the volatility and thermal stability of the material. Gas-phase molecules are best analysed with electron impact ionisation (EI+). EI+ of an organic molecule M results in the formation of a molecular ion $M^{+}\bullet$ by removal of an electron:-

$$M + e^{-} \rightarrow M^{+}\bullet + 2e^{-}$$

During the ionisation, a certain amount of internal energy is transmitted to the molecule. This quantity of energy is variable, and its magnitude determines whether

one or more fragmentation reactions occur. The most likely reactions are those which have low activation energy, leading to relatively stable ionic or neutral species:-

$M^{+}\bullet$	$\rightarrow$	ionic fragment	+	neutral fragment
		$(BC^{+}\bullet)$		(radical, atom, molecule)
		$\downarrow$		
		ionic fragment	+	neutral fragment
		$\downarrow$		
		etc		

The successive fragmentation reactions generally lead to ions and radicals consisting of a single atom, or group of atoms. During fragmentation, the charge is located on the fragment with the lowest ionisation potential. Therefore in the fragmentation of $BC^{+}\bullet$ into B^{+} and C•, or into B• and C^{+}, if the ionisation potential of B is lower than that of C, then the major fragmentation path will be into B^{+} and C•; similarly, for fragmentation of an ion BC^{+}, under the same conditions, the most likely fragmentation would be into B^{+} and C, not into B and C^{+}.

Stable fragments can also be produced by a change in the relative positions of the atoms in a molecule, followed by decomposition of the ion, so called rearrangement reactions.

5.2.2 Ifosfamide and GC–MS

In terms of technique, GC–MS has the advantage of removing troublesome background noise, allowing the accurate determination of multiple metabolites. Prior to introduction into the MS, the metabolites are separated by GC. Ifosfamide undergoes cyclization in alkaline media resulting in the formation of an aziridine. This reaction lowers the efficiency of extraction of ifosfamide from an alkaline solution, but is totally reversed at acidic pH. Ifosfamide itself is sufficiently thermally stable to enable gas chromatographic determination without derivatisation

(Lambrechts et al 1991). In contrast, the isomer cyclophosphamide is perfectly stable under alkaline conditions (no aziridine structure is possible), but needs derivatisation for GC analysis, otherwise partial thermal cyclization in the injector produces two separate product peaks. GC generally requires derivatization of metabolites with trifluoroacetic or heptafluorobutyric anhydride. Several metabolites of ifosfamide form different derivatives with trifluoroacetic anhydride (TFAA). The trifluoroacetyl derivatives of oxazaphosphorine metabolites can be measured with picogram sensitivity by single ion monitoring (SIM) GC–MS. This chapter describes the development of a GC–MS method for analysing ifosfamide and its metabolites.

5.3 Methods

Ifosfamide, the two possible dechloroethyl metabolites, KIPA, CIPA, IPM, CEA and OXA were determined by electron capture ionization and electron impact mass spectrometric detection of the trifluoroacetyl derivatives. Procedures analogous to those used in the analysis of cyclophosphamide and its metabolites were employed (Momerency et al 1994).

5.3.1 Chemicals and extraction procedure

Reference compounds of most of the metabolites were provided by ASTA-Werke, Degussa Pharma Gruppe, Bielefeld, Germany. Water and organic solvents used in the procedure were of HPLC quality. TFAA was obtained from Janssen Chimica, Geel, Belgium. A 0.05 M citrate – hydrochloric acid mixture was employed as pH 4 buffer. SEP-PAK solid phase extraction cartridges containing 500 mg of trifunctional C_{18} reversed phase material, supplied by Millipore, were used on a vacuum manifold station (Momerency et al 1994).

To extract the acidic metabolites, 500 µl of citrate buffer pH 4 was added to 500 µl of plasma, together with 25 µl internal standard (IS) solution (cyclophosphamide,

CPA) (table 5.1). An IS was used to compensate for differences in extraction efficiency and chromatographic variations after derivatisation. This mixture was passed over Sep Pak C_{18} solid phase extraction cartridges, previously conditioned with 5 ml methanol. The cartridges were then washed with 5 ml water, and residual water removed by blowing 10 ml air through the cartridges. The plasma components were eluted with 5 ml methanol, which was evaporated under nitrogen, and the residue subjected to the derivatisation procedure.

To measure the basic metabolites, CEA and OXA, N-chloroethyl-1, 3-oxazolidine-2-one (the corresponding oxazolidinone of cyclophosphamide, CEOX) was used as the IS. The metabolites were isolated by ethyl acetate extraction after dilution with 0.05 M borate – sodium hydroxide buffer of pH 10.

5.3.2 Derivatisation

Derivatization comprised two steps; the methylation of the acidic groups of CIPA and IPM followed by trifluoroacetylation of unhindered phosphoramide groups, using a large excess of the reagents (Momerency et al 1994). Diazomethane in ether was added to the residue and the solvent evaporated under nitrogen (methylation of acids). The residue was re-dissolved in 300 µl ethyl acetate and 300 µl trifluoroacetic anhydride was added. This solution was held at 70°C for 2 hours (acylation of amides). The solvent and the excess reagent were again evaporated under nitrogen and the residue re-dissolved in 250 µl ethyl acetate. These samples were used for quantitative GC analysis. For the basic metabolites, after centrifugation and evaporation of the upper layer under nitrogen, the solution was derivatised with trifluoroacetic anhydride only (there are no acidic groups to methylate).

Determination of IPA, KIPA, IPM, CIPA, and 2- and 3-DCEI	**Determination of CEA and OXA**
0.5 ml plasma	0.5 ml plasma
↓ + IS CPA	↓ + IS CEOX
+ 0.5 ml buffer pH 4	+ 0.5 ml buffer pH 10
↓	↓
1 ml diluted plasma, vortex	1 ml diluted plasma, vortex
↓	↓
C_{18} column (500 mg)	extraction with ethyl acetate (3 ml + 2 ml), centrifugation
↓	↓
elution with 5 ml methanol; complete evaporation	partial evaporation
↓	↓
dry residue; + diazomethane in ether for 15 min; evaporation	derivatisation; + 0.3 ml ethyl acetate + 0.3 ml TFAA at 70 °C for 2 hours
↓	↓
derivatisation; + 0.3 ml ethyl acetate + 0.3 ml TFAA at 70 °C for 2 hours	partial evaporation
↓	↓
complete evaporation; dissolve in 250 µl ethyl acetate	dissolve in 250 µl ethyl acetate
↓	↓
GC–MS	GC–MS

Table 5.1. Schematic diagram of the enrichment procedure.

5.3.3 Gas chromatographic – mass spectrometric analysis

Gas chromatographic separations were performed on a Carlo-Erba Mega Series 5160 chromatograph, equipped with a 25 m x 0.25 mm i. d. fused silica capillary column coated with a 0.2 mm film of Sil-13 (methyl phenyl siloxane; Chrompack, Middelburg, Holland). Through a short metal capillary transfer line maintained at 250°C, the fused silica GC column was connected to the combined electron impact - chemical ionisation source of a Fisons Trio-2A quadrupole mass spectrometer (Fisons Instruments, UK) maintained at 180°C. EI+ was performed with a trap current of 200 mA, an electron energy of 70 eV, and an ion repeller potential of 1 V. Chemical ionisation (CI–) was carried out using a source current of 800 mA, an electron energy of 40 eV, and an ion repeller potential of 0 V. Multiple ion monitoring (MIM) was performed on extracts of spiked plasma and patient plasma. Linear temperature programming of the GC column enabled selection of retention windows in the chromatogram, during which specific m/z values from the mass spectra of the metabolites were measured.

The temperature programme for measuring IPM, 2-DCEI, 3-DCEI, IPA, KIPA and CIPA was 150°C to 240°C, and for CEA and OXA 80°C to 150°C, both increasing by 6°C min^{-1}. With the exception of CEA and OXA, all compounds were measured by CI– with electron capture. CEA and OXA were analysed using classical EI+. SIM was used throughout.

5.3.4 Calibration curves

Standard solutions of known concentrations of ifosfamide and its metabolites (5 ng ml^{-1} to 100 ng ml^{-1} and 0.1 μg ml^{-1} to 20 μg ml^{-1} of blank plasma) were submitted to the same extraction and derivatisation procedures as the patient plasma samples. These solutions were co-injected in the GC during the same sequence of measurements as the patient plasma samples, thus keeping the effects of variations in ion source contamination of the MS as low as possible. The ratio of peak area for a

given metabolite to that for the internal standard was plotted against the concentration of that metabolite in the original spiked plasma sample to produce a calibration curve. Linear regression analysis of the data points (ratio of metabolite peak area to internal standard peak area, y, against metabolite concentration, x, in the original plasma) produced a best fit; e.g. the regression equation for IPM was $y = (0.01579 \pm 0.00037)\, x + (0.00125 \pm 0.00022)$ ($n = 6$).

The intra-assay reproducibility, expressed as relative standard deviation, was approximately 3%, and the inter-assay reproducibility ranged from 5% to 10% for sets of four independent extractions.

5.4 Results

5.4.1 Derivatisation

The derivatisation procedures employed generate the bis-trifluoroacetyl derivative of 3-DCEI. On standing in the presence of ether or chloroform, an equilibrium mixture of the two mono derivatives of 3-DCEI is formed. 2-DCEI was measured as the monotrifluoroacetyl derivative. The bis-trifluoroacetyl derivative of 2-DCEI was not detected.

CIPA has two identical phosphoramidic functions, which can be further substituted. Theoretically both can be trifluoroacetylated, but in practice only one is actually transformed, probably because of the effect of steric hindrance in the mono derivative. In the corresponding carboxy metabolite of cyclophosphamide, a further loss of hydrogen chloride is observed yielding a single cyclic derivative as the only chromatographic peak (Momerency et al 1994). A similar process does not occur with CIPA. The metabolite 4-ketocyclophosphamide cannot be derivatized by reaction with TFAA because of a mesomeric effect involving the endocyclic amidic nitrogen atom. In contrast, trifluoroacetylation of KIPA occurs smoothly on the exocyclic nitrogen atom. KIPA does not react with diazomethane solution. Unlike

the cyclophosphamide mustard (CPM), which decomposes completely to nor-nitrogen mustard (NNM) during attempts to derivatize with TFAA, IPM does not decompose during the enrichment and derivatization steps, and can easily be recovered from plasma. Methylation occurs only on the phosphoramidic acid function and trifluroacetylation only on one of the equivalent phosphoramidic nitrogen atoms (figure 5.1). The derivative has an acyclic structure, and the subsequent loss of hydrogen chloride with the formation of a cyclic product, seen with several cyclophosphamide metabolites (Momerency et al 1994), is not observed with this derivative. The structures of the metabolite derivatives are given in figure 5.2.

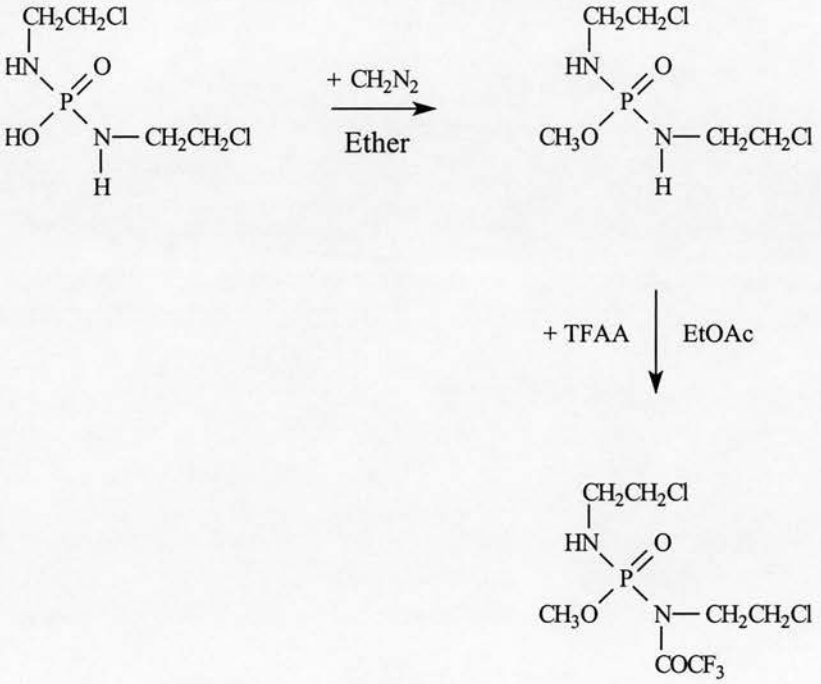
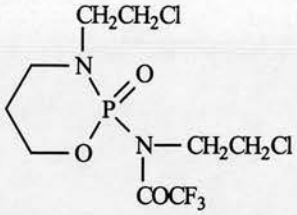
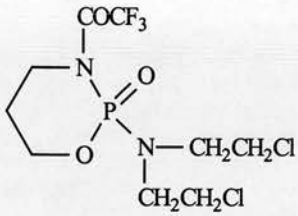


Figure 5.1. Derivatisation of IPM.

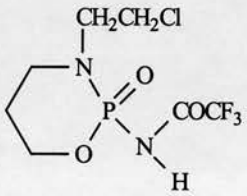
The mass spectrum of trifluoroacetylated ifosfamide is shown in figure 5.3. Trifluoroacetyl ifosfamide produces an EI+ mass spectrum similar to that of the cyclophosphamide derivative, with a base peak at m/z = 307/309 for the fragment $(M - CH_2Cl)^+$. The characteristics of the EI+ and CI– mass spectra of the metabolite



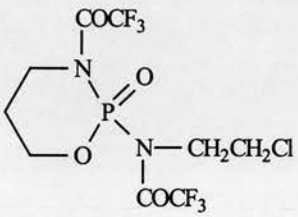
TFA-IPA



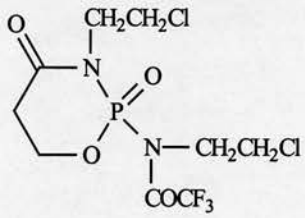
TFA-CPA



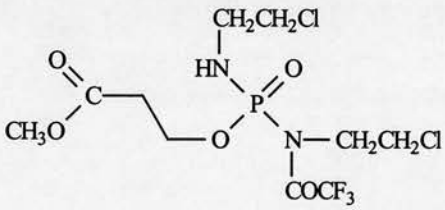
TFA-2-DCEI



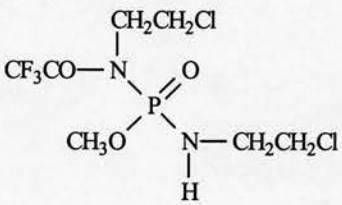
Bis TFA-3-DCEI



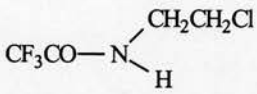
TFA-KIPA



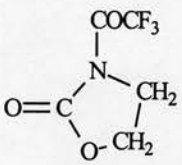
TFA-CIPA-Me



TFA-IPM-Me



TFA-CEA



TFA-OXA

Figure 5.2. Structures of the stable metabolite derivatives of ifosfamide.

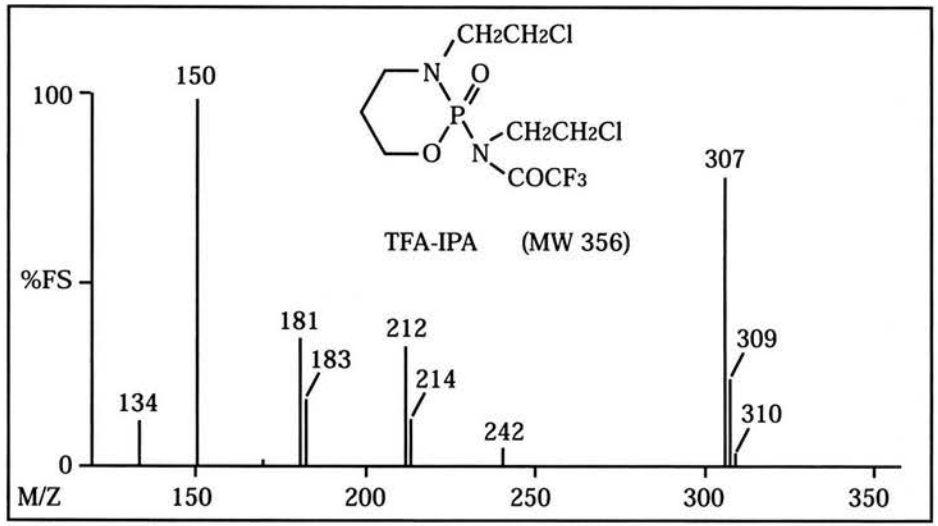


Figure 5.3. Mass spectrum of trifluoroacetylated ifosfamide (TFA-IPA).

derivatives are shown in table 5.2. EI+ and CI– mass spectra of selected metabolite derivatives are presented in figures 5.4 and 5.5.

5.4.2 Recovery of ifosfamide metabolites from spiked plasma samples

Extraction yields of ifosfamide metabolites using the C_{18} solid phase extraction procedure at acidic pH were determined. More extensive data is available for cyclophosphamide metabolites using liquid extraction with ethyl acetate or chloroform, and solid phase isolation with either silica gel or C_{18} at pH 4, 7 or 10 (Momerency et al 1994). One would expect the extraction efficiencies of ifosfamide metabolites to be similar to those of cyclophosphamide metabolites, as shown for the parent compounds at acidic pH with C_{18} solid phase extraction (table 5.3).

The observed extraction efficiencies at pH 4 were generally higher than 90% for both dechloroethyl metabolites, the keto metabolite, and for the parent compounds ifosfamide and cyclophosphamide. The recovery of CIPA was somewhat lower at approximately 75%. Both IPM and CPM are stable in a weakly acidic medium. As CPM decomposed readily during attempts at derivatization, the efficiency with which it was extracted was measured by thermospray LC–MS (SIM) of the underivatized form of the molecule. The ion monitored was the $(M + NH_4 - HCl)^+$ at m/z 202. The recovery of IPM from C_{18} columns is low, only about 50%, and comparable with that of CPM, but is still 10% higher than the value given in the literature for extraction of CPM using Amberlite XAD-4 columns (Jardine et al 1978).

The isolation of the alkaline CEA and OXA metabolites was performed by liquid extraction with ethyl acetate at pH 10; recoveries were 85% and 90% respectively, an improvement on the solid phase procedure at pH 7. No further decomposition of the metabolites was observed during storage of spiked plasma samples at -20°C for at least three weeks.

Compound	MW	Fragment	Interpretation
EI+			
TFA-IPA	356	307/309	$(M - CH_2Cl)^+$
TFA-CPA (IS)	356	307/309	$(M - CH_2Cl)^+$
TFA-2-DCEI	294	245	$(M - CH_2Cl)^+$
bis-TFA-3-DCEI	390	341	$(M - CH_2Cl)^+$
TFA-CIPA-Me	402	324/326	$(M - NHCH_2CH_2Cl)^+$
TFA-KIPA	370	335/337	$(M - Cl)^+$
TFA-CEA	175	126	$(M - CH_2Cl)^+$
TFA-OXA	183	114	$(M - CF_3)^+$
TFA-IPM-Me	330	281/283	$(M - CH_2Cl)^+$
CI–			
Group I			
TFA-CPA	356	320/322	$(M - HCl)^-$
TFA-2-DCEI	294	274/276	$(M - HF)^-$
TFA-OXA	183	183	$(M)^-$
TFA-CIPA-Me	402	185	?
Group II			
TFA-IPA	356	293/295	$(M - CH_2CH_2Cl)^-$
bis-TFA-3-DCEI	390	327	$(M - CH_2CH_2Cl)^-$
TFA-KIPA	370	307/309	$(M - CH_2CH_2Cl)^-$
TFA-CEA	175	112	$(M - CH_2CH_2Cl)^-$
TFA-IPM-Me	330	267/269	$(M - CH_2CH_2Cl)^-$

MW – Molecular Weight

Table 5.2. Characteristic fragments of the EI+ and CI– mass spectra of ifosfamide metabolite derivatives.

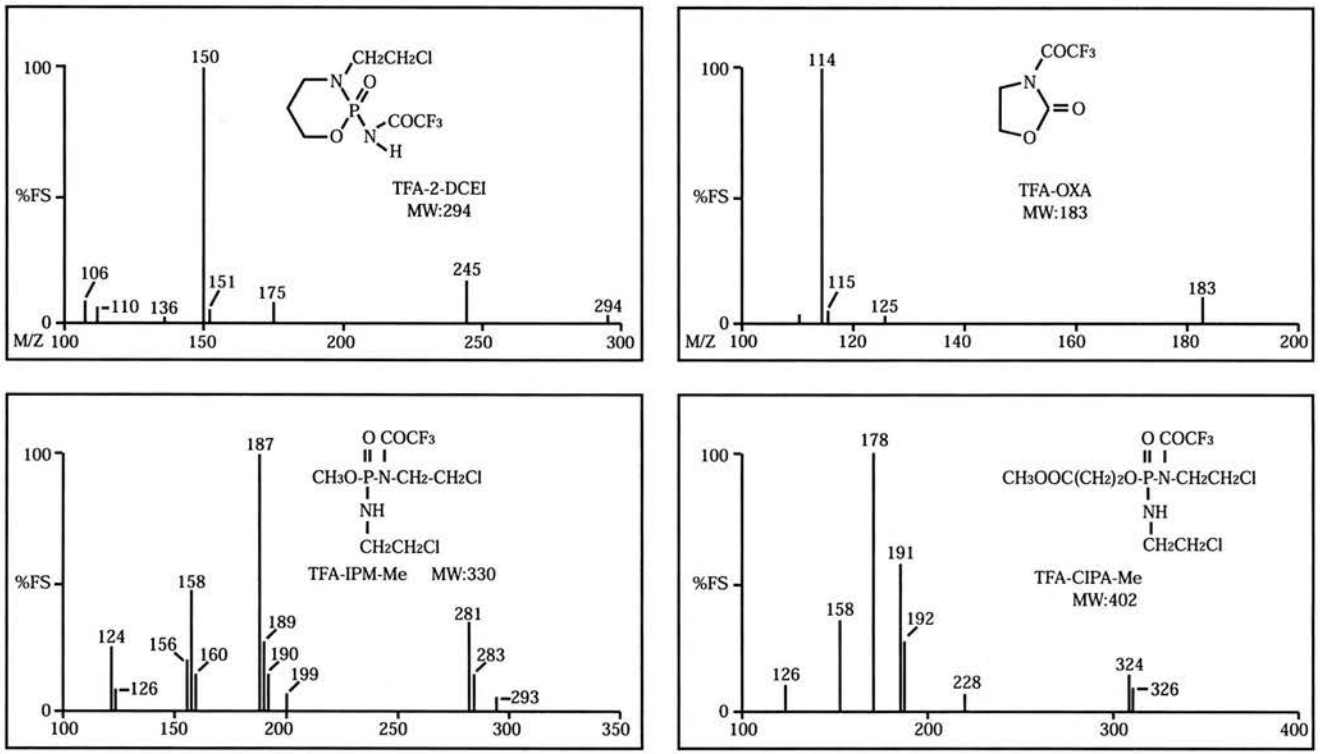


Figure 5.4. Electron impact mass spectra of TFA-2-DCEI, TFA-OXA, TFA-IPM-Me, and TFA-CIPA-Me.

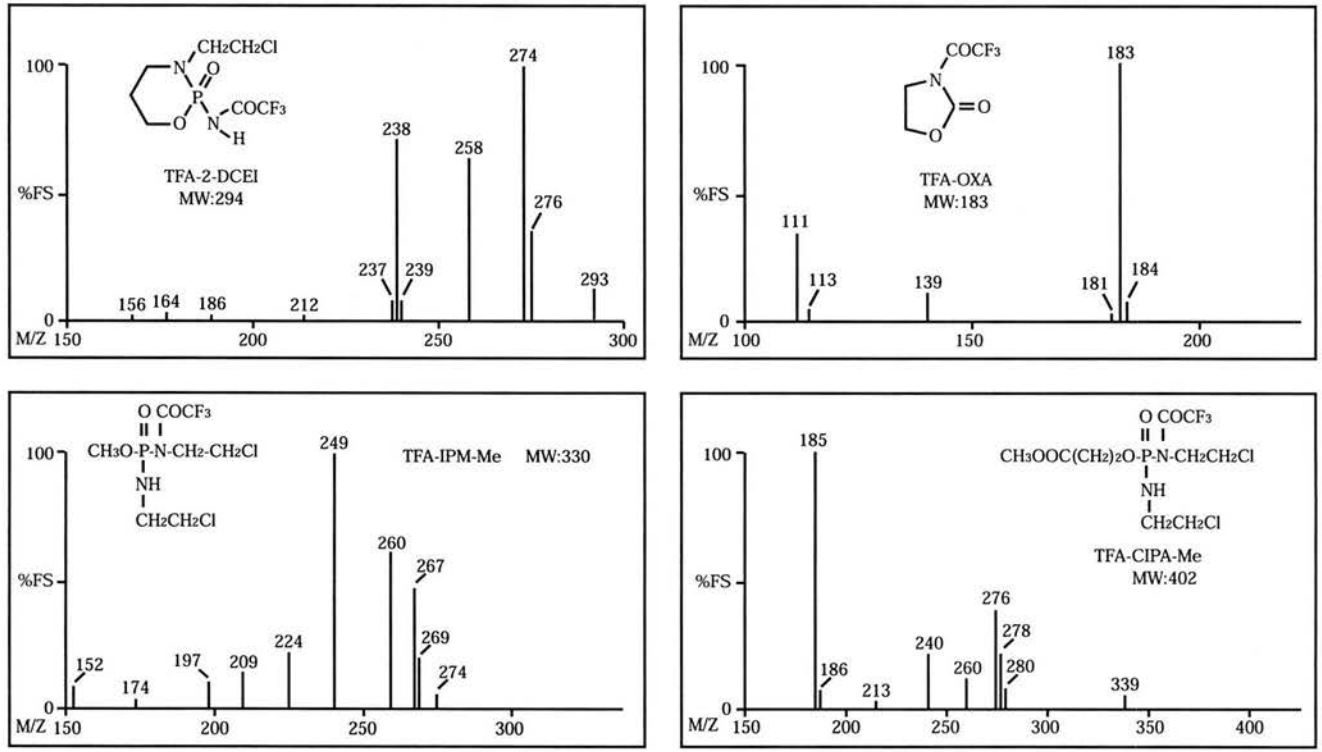


Figure 5.5. Electron capture chemical ionization mass spectra of TFA-2-DCEI, TFA-OXA, TFA-IPM-Me, and TFA-CIPA-Me.

Cyclophosphamide	Ifosfamide
CPA: 93.2% ± 3.6%	CPA (IS): 93.2% ± 3.6%
IPA (IS): 99.7% ± 3.7%	IPA: 99.7% ± 3.7%
DCEC: 99.5% ± 2.8%	3-DCEI: 96.8% ± 4.1%
	2-DCEI: 92.7% ± 6.4%
KETO-CPA: 90.3% ± 2.6%	KIPA: 91.5% ± 3.8%
CARBOXY-CPA: 82.5% ± 3.9%	CIPA: 74.4% ± 1.9%
ALCO-CPA[a]: 75.9% ± 5.0%	–
CPM[b]: 56.3% ± 4.0%	IPM: 49.8% ± 4.2%

a Alco-IPA was not available as a reference compound
b Measured by thermospray LC–MS

Table 5.3. Recoveries of ifosfamide and cyclophosphamide metabolites using C_{18} solid phase extraction at pH 4.

In the procedure used to measure the stable cyclophosphamide metabolites in plasma, it was observed that CI–, using methane as quenching gas, provided much better sensitivity than EI+ for many trifluoroacetylated metabolite derivatives (Momerency et al 1994). The same is true for the ifosfamide metabolites.

During CI–, two different types of fragmentation of the ifosfamide and cyclophosphamide metabolites occurs; this is illustrated by subdivisions I and II in table 5.2. The derivatives in group II contain a special functional group which fragments by the loss of a CH_2CH_2Cl substituent, e.g. the IPM derivative with a characteristic peak at m/z 267/269. A tentative mechanism is shown in figure 5.6. A tentative assignment for the base peak fragment ion from the CIPA derivative at m/z = 185 cannot yet be proposed. However, the fragment is of diagnostic value for the CIPA derivative, and is used in quantitative work.

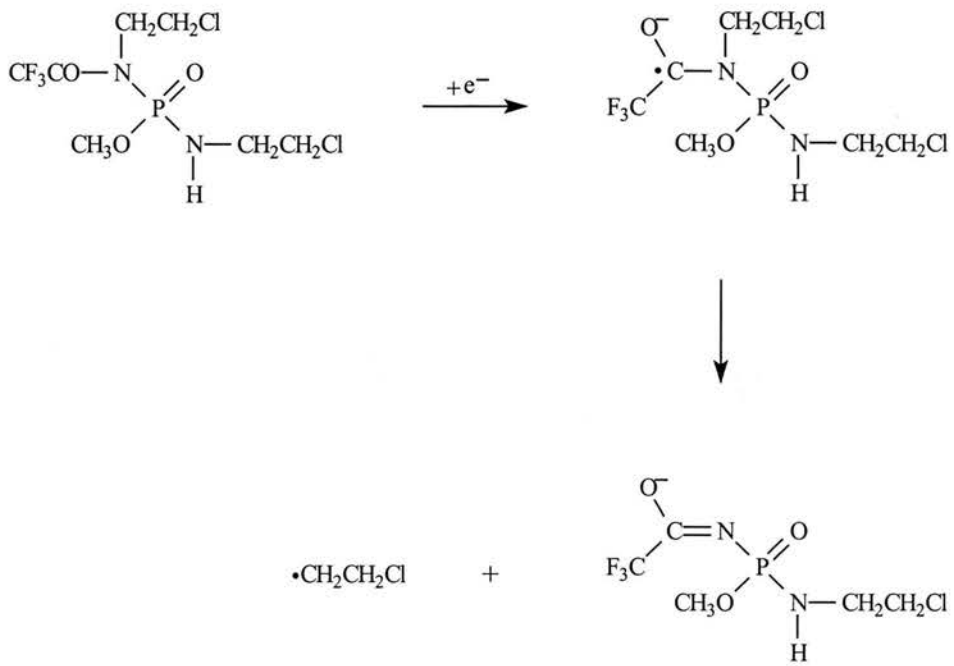


Figure 5.6. Fragmentation of the derivative of IPM in electron capture chemical ionization MS.

Table 5.4 lists the retention times of the ifosfamide metabolite derivatives in increasing order. Separate retention windows can be easily established for SIM of most ifosfamide metabolites. However, since two regions of partial overlap exist, in which retention times differ by less than one minute, MIM was applied in these regions, giving complete separation using the appropriate specific ion masses, although the ultimate detection sensitivity was reduced by a factor of two to three. The MIM time intervals in order of increasing retention times are listed in table 5.5.

Injection of a 1 µl aliquot from 50 µl of final derivatised extract enabled the determination of plasma metabolite concentrations in the range of 3 ng ml^{-1} to 20 µg ml^{-1}.

Ifosfamide	t_R (min)
TFA-IPM-Me	6.71
bis-TFA-3-DCEI	7.40
TFA-2-DCEI	8.26
TFA-IPA	12.63
TFA-KIPA	12.93
TFA-CIPA-Me	14.03
TFA-CPA (IS)	14.83

Table 5.4. Retention times (t_R) of ifosfamide and its metabolites on Chrompack Sil-13; (Linear temperature programme from 150°C to 240°C at 6°C min^{-1}).

Electron capture CI– was the most sensitive technique for the quantitative measurement of ifosfamide, 3-DCEI, KIPA and CIPA; no substantial difference was observed between EI+ and CI– sensitivity for 2-DCEI and IPM. All these metabolites can therefore be measured with equally high sensitivity by electron capture CI– (a typical MIM chromatogram is shown in figure 5.7 a). CEA and OXA were measured with greater sensitivity by EI+ (a typical SIM chromatogram is shown in figure 5.7 b); it was not possible to detect the parent ion of TFA-CEA, but intense peaks were observed using the mass fragment m/z 126.02.

Metabolite derivative	Specific ion	m/z	Retention window (min)
CI–			
TFA-IPM-Me	$(M - CH_2CH_2Cl)^-$	266.99	2.0 - 10.0
bis-TFA-3-DCEI	$(M - CH_2CH_2Cl)^-$	327.00	2.0 - 10.0
TFA-2-DCEI	$(M - HF)^-$	274.00	2.0 - 10.0
TFA-IPA	$(M - CH_2CH_2Cl)^-$	293.01	10.0 - 13.5
TFA-KIPA	$(M - CH_2CH_2Cl)^-$	306.99	10.0 - 13.5
TFA-CIPA-Me	$(M - ?)^-$	185.01	13.5 - 17.0
TFA-CPA (IS)	$(M - HCl)^-$	320.03	13.5 - 17.0
EI+			
TFA-CEA	$(M - CH_2Cl)^+$	126.02	2.0 - 6.0
TFA-OXA	$(M - CF_3)^+$	114.02	6.0 - 12.0
CEOX (IS)	$(M - CH_2Cl)^+$	100.04	6.0 - 12.0

Table 5.5. Multiple ion monitoring programmes for ifosfamide metabolites; dwell time 0.08 s; span 0.02 a. m. u.

Measurement of the labile primary metabolite 4-hydroxyifosfamide as a separate peak is not possible. Since the hydroxy structure is stabilised at a weakly acidic pH (Ikeuchi and Amano 1985), and at least in the case of cyclophosphamide easily decomposes to CPM during the C_{18} solid phase extraction procedure (Watson et al 1985), it is probable that the IPM concentration measured actually represents the sum of the free mustard, 4-hydroxyifosfamide, and aldoifosfamide.

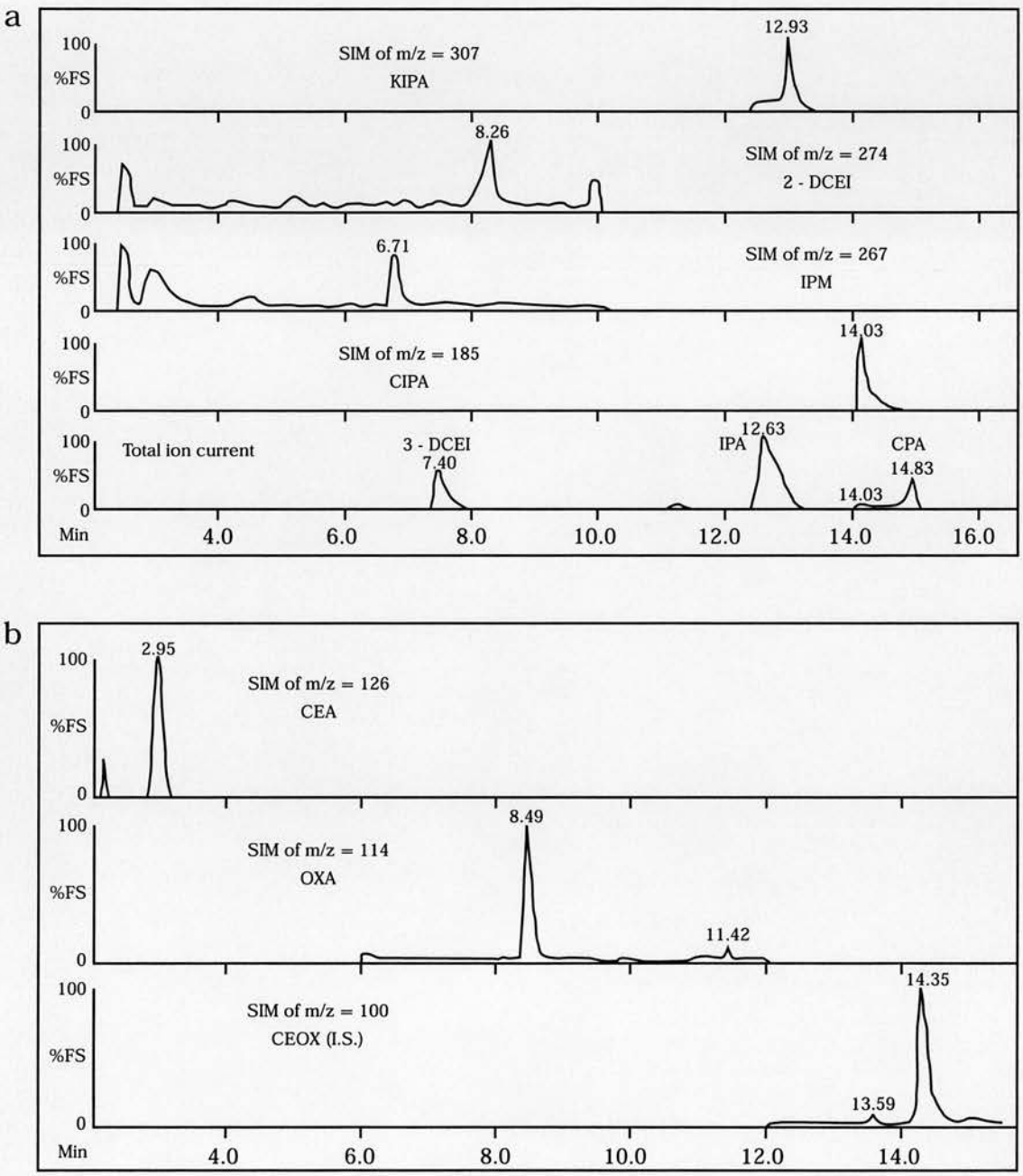


Figure 5.7. CI– SIM and MIM chromatograms (upper, a), and EI+ SIM chromatogram (lower, b), of a plasma extract.

5.5 Conclusion

This chapter describes a new analytical method for the determination of ifosfamide and its metabolites in human biofluids, using GC–MS. The method was employed to analyse these compounds after oral and intravenous administration of ifosfamide.

Chapter 6

Pharmacokinetic Principles

6.1 Introduction

Following the administration of a drug, the body can be regarded as a collection of separate compartments, each containing a fraction of the administered dose. The transfer of compounds from one compartment to another is associated with a rate constant (k), the magnitude of which determines how fast the transfer occurs. The moment a drug reaches the blood stream it is subject to both distribution and elimination. Rate constants associated with distribution are much larger than those of drug elimination. Therefore drug distribution throughout the body is usually complete while most of the dose is still in the body. Some drugs attain distribution equilibrium quickly, essentially before any of the dose is eliminated, and in such cases the body appears to have the characteristics of a single compartment. However this is too simple for most drugs. Concentrations in plasma measured shortly after intravenous administration reveal a distinct distributive phase, i.e. a measurable fraction of the dose is eliminated before distribution equilibrium is reached, and a multicompartmental system effectively exists. Two or three compartments are usually used to describe the time course of a drug in plasma: the rapidly equilibrating, or central, compartment; and one or two slowly equilibrating, or peripheral, compartments.

6.2 Physical significance of plasma drug concentration

The degree of distribution and binding is a function of the physical and chemical properties of a drug. At distribution equilibrium, drug concentrations in different parts of the body are rarely equal. Some sites such as the CNS, or fat, are poorly accessible to some drugs. Other tissues may have a great affinity for the drug and bind it avidly.

However once a drug attains distribution equilibrium, its concentration in the plasma reflects distribution factors, and the simple relationship between amount of drug in the body (A) and drug concentration in plasma (C) applies:-

$$A = Vd\,C \qquad 6.1$$

where Vd is the apparent volume of distribution.

In most situations Vd is independent of drug concentration. Doubling the amount of drug in the body, e.g. by doubling the intravenous dose, usually results in a doubling of drug concentration in plasma. This is called dose proportionality, and is often used as an indicator of linear pharmacokinetics.

Vd is usually a characteristic of the drug rather than the biological system, although it may be changed by disease and other factors. Vd rarely corresponds to the volume of plasma, extracellular fluid or total body water, and is usually not an anatomical volume, but a reflection of drug distribution and a measure of the degree of drug binding. Acidic drugs are often preferentially bound to plasma proteins rather than to extravascular sites. Although these drugs distribute throughout body water, their Vd values are small. Many basic drugs are more extensively bound to extravascular sites than to plasma proteins, and the value of Vd for these drugs is large.

6.3 Pharmacokinetic considerations of plasma drug concentration

6.3.1 *Intravenous administration*

It is not necessary to consider absorption when a drug is given by rapid intravenous injection. As soon as the drug is administered, it undergoes distribution and is subject to one or more elimination pathways. The amount of drug in the body and the drug concentration in plasma decrease continuously after injection. At the same time there is

continuous formation of metabolites and continuous excretion of drug and metabolites. Eliminated products accumulate while drug levels in the body decline.

Most drugs distribute rapidly, so that shortly after intravenous injection, distribution equilibrium is reached. Drug elimination at distribution equilibrium is usually described by first-order kinetics, i.e. the rate of the process is proportional to the amount or concentration of substrate or drug in the system. As drug concentration falls the elimination rate falls in parallel. The proportionality constant relating rate and amount, or concentration, is called a rate constant.

$$\log C = \log C_0 - kt/2.303 \qquad 6.2$$

where C = concentration; t = time; and k = the first-order elimination rate constant. The term C_0 is the intercept on the log concentration axis, on extrapolation of the linear segment to t = 0.

Equation 6.2 indicates that a plot of log C against t will be linear, once distribution equilibrium is reached. The linear portion of the semi-logarithmic plot of C against t has a slope corresponding to -k/2.303, and an intercept on the y-axis (i.e. at t = 0) corresponding to C_0.

Although it is possible to calculate the elimination rate constant from the slope of the line, it is much easier to determine k by:-

$$k = 0.693 / t_{1/2} \qquad 6.3$$

where $t_{1/2}$ is the half-life of the drug and is determined directly from the plot.

In a first-order process the half-life is independent of dose or initial plasma concentration. The half-life of a drug can often be related to the duration of clinical effect and the frequency of dosing.

An important indicator of exposure to a cytotoxic is the total area under the plasma concentration-time curve. Sometimes studies are not carried out long enough to allow drug concentrations to fall to negligible levels. The total AUC then cannot be determined directly, only the partial AUC. A widely used method is to determine the AUC from t = 0 to the last sampling time (t*), by means of the trapezoidal rule, and to estimate the missing area by the equation:-

$$\text{Area from } t^* \text{ to } \infty = C^* / k \qquad 6.4$$

where C* is the drug concentration at t = t*, and k is the apparent first-order elimination rate constant.

This area must be added to the area calculated from time zero to t* (AUC_t) to obtain the total AUC.

6.3.2 Short term constant rate intravenous infusion

Drug concentration in the plasma increases during an infusion. When the entire dose has been infused at time T, drug concentration reaches a maximum and declines thereafter. The declining drug concentration is described by:-

$$\log C = \log Cmax - (kt' / 2.303) \qquad 6.5$$

where $t' = t - T$.

Equation 6.5 applies when distribution equilibrium is essentially reached by the end of the infusion. A semi-logarithmic plot of C (post-infusion drug concentration in plasma) against t' yields a straight line, from which the half-life and elimination rate constant can be estimated. The maximum drug concentration in plasma is always lower after

intravenous infusion than after bolus injection of the same dose. The more slowly a fixed dose of a drug is infused, the lower the value of Cmax.

6.3.3 Extravascular administration

A more complex drug concentration-time profile is observed after oral administration because absorption is not instantaneous, nor does it occur at a constant rate. The rate of change of the amount of drug in the body is a function of both the absorption rate and the elimination rate. When the absorption rate is greater than the elimination rate, the amount of drug in the body, and the plasma drug concentration, increase with time. Conversely, when the amount of drug remaining at the absorption site is sufficiently small so that the elimination rate exceeds the absorption rate, the amount of drug in the body, and the plasma drug concentration, decrease with time. The maximum concentration after drug administration occurs at the moment the absorption rate equals the elimination rate. The faster a drug is absorbed, the higher the maximum concentration in plasma after a given dose, and the shorter the time after administration when the peak is observed.

First-order drug input assumes that the rate of drug entry is proportional to the amount of drug remaining to be absorbed. The proportionality factor is the absorption rate constant (KA). Absorption from the gastrointestinal tract may be defined by:-

$$\text{Rate in} = KA \times G(t) \qquad 6.6$$

where G(t) is the amount of the dose remaining in the gastrointestinal tract at time t.

Many drugs have a KA of 0.5 hr^{-1} to 2 hr^{-1}. This corresponds to an absorption half-life ($\log_n [2] / KA$) of 20 minutes to 1.5 hours. If the disposition half-life of the drug is long relative to absorption, then the time of peak drug concentration will be one to five hours, i.e. approximately three absorption half-lives. Good initial estimates of

KA can be made by calculating the absorption half-life as one third of the time of peak concentration, and deriving KA from 0.7 / (absorption half-life) ($\log_n$ [2] is approximately 0.7).

When absorption is complete, the rate of change of the amount of drug in the body equals the elimination rate. The section of the plasma drug concentration-time curve commencing at the time absorption has ceased is called the post-absorptive phase. During this phase the decline in drug concentration follows first-order kinetics. The absorption rate of a drug from the gastrointestinal tract varies with the rate of gastric emptying, the time of administration with respect to meals, the physical and chemical characteristics of the drug, and the dosage form, as well as other factors. Similarly, the amount of an oral dose of a drug that is absorbed depends on biological, drug, and dosage form considerations. The bioavailability of a drug is defined as its rate and extent of absorption.

6.3.4 Repetitive dosing

During repetitive oral administration of the same dose at regular intervals, plasma concentrations during a dosing interval first increase, and then decrease as a result of absorption, distribution and elimination. The magnitude of the concentration difference in a dosing interval depends on the rates of absorption and distribution, and on the half-life of the drug; this concentration difference increases with increasing absorption rate and decreasing half-life. A simple way of describing the steady state is to consider the average drug concentration (Css).

6.4 Noncompartmental analysis

This is based on the theory of statistical moments (Yamaoka et al 1978). The zero moment of a plasma drug concentration-time curve is the total AUC from time zero to infinity. The first moment is the total AUC resulting from a plot of the product of drug

concentration and time against time. The total area under the C.t against t plot is termed the AUMC, or area under the first moment curve.

The ratio of AUMC to AUC for any drug is a measure of its mean residence time (MRT).

$$MRT = AUMC / AUC \qquad 6.7$$

MRT calculated after intravenous administration is the statistical moment analogy to drug half-life. Like half-life, MRT is a function of both distribution and elimination.

Clearance is a function of both the intrinsic ability of certain organs, such as the kidneys and liver, to excrete or metabolise a drug and the rate of blood flow to these organs. The total body clearance of a drug from the blood is equal to the ratio of the overall elimination rate of the drug to the drug concentration in blood, where the overall elimination rate is the sum of the elimination processes occurring in all organs. The ratio of the overall elimination rate of a drug to its concentration in the blood is equal to the ratio of the amount of drug ultimately eliminated to the total area under the drug concentration-time curve. After intravenous administration the amount eliminated is equal to the dose, and clearance can therefore be expressed as:-

$$CL = dose / AUC \qquad 6.8$$

One of the most useful properties of statistical moments is that they permit the estimation of a volume of distribution that is independent of drug elimination. Using these methods, the apparent volume of distribution at the steady state (Vss) is given by the product of the intravenous bolus dose and the ratio of AUMC to AUC squared. It represents the proportionality constant relating the amount of drug in the body at the steady state after prolonged constant rate intravenous infusion, or repetitive administration, to the drug concentration, or average drug concentration, at that time. Vss is independent of drug elimination, and reflects solely the anatomical space occupied by a drug and the relative degree of drug binding in the blood and

extravascular space. Estimation of Vss does not require data obtained at the steady state; this distribution parameter can be calculated after a single dose of drug.

$$Vss = \text{iv dose} * AUMC / AUC^2 \qquad 6.9$$

where AUMC is the total area under the first moment curve.

Although equation 6.9 applies only to dosing by intravenous bolus, the relationship can be modified to accommodate other routes of administration. If a drug is given by a short-term constant rate intravenous infusion, then

$$Vss = [\text{infused dose} * AUMC / AUC^2] - [\text{infused dose} * (T)/2\ (AUC)] \qquad 6.10$$

where T is the duration of infusion.

6.5 Multiple compartmental analysis

Following intravenous bolus administration many drugs distribute sufficiently slowly so that a significant fraction of the dose is eliminated before distribution equilibrium is achieved. The data cannot be described by a single exponential expression (i.e. a single compartment). At the outset drug concentrations decline rapidly, and these early phases are typically associated with distribution. Ultimately, a linear relationship between log concentration and time is observed. Irrespective of the complexity of the model, drug concentrations in the plasma decline in a first-order manner once distribution equilibrium is achieved. This final phase is known as the terminal phase, and it is often assumed that the drug leaves the site of measurement only by elimination during this phase. The rate constant describing this first-order portion of the curve is usually termed β. The log-linear region of the curve will have a slope equal to $-\beta / 2.303$. Therefore for drugs that require a multiple compartmental description, a terminal half-life may be defined as:-

$t_{1/2} = 0.693 / \beta$ 6.11

Elimination occurs during every phase, and the half-life of each phase is a function of distribution, re-distribution and elimination processes. As drug elimination becomes more efficient, and clearance increases, then elimination half-life decreases in an inversely proportional manner. However changes in distribution also influence half-life if clearance remains constant. The Vd of ifosfamide increases in older patients, leading to an increased half-life (Lind et al 1990a). This results in lower concentrations at the site of measurement for a longer duration, possibly altering efficacy and toxicity. If clearance and Vd both change by the same proportion, half-life does not change, but lower concentrations are observed at the site of measurement.

The half-life always reflects both distribution and elimination, irrespective of the model used. In a single compartment model, elimination is represented by clearance and distribution by Vd. Multiphasic profiles possess kinetically distinct components in the concentration-time profile. The frequency and duration of pharmacokinetic sampling determines whether such multiple phases can be identified, and the associated half-lives measured. The half-life associated with a particular phase describes the observed decay only if the contributions from the other phases are negligible. Each half-life depends to some extent on every rate constant. If all phases are important, one subset of pharmacokinetic parameters is unable to describe the full time course of cytotoxic administration. Cisplatin pharmacokinetics are usually described by biphasic models, but recently developed sensitive analyses have led to the identification of a very late terminal phase with a half-life of 720 days, commencing 900 days after the last administration (Schierl et al 1995).

Various statistical considerations are useful in minimising the problems associated with model selection, but they do not overcome them. Studies with a single drug in a group of patients may result in some patients requiring a two compartment model to describe the pharmacokinetics of the drug, whereas others require a three compartment model.

6.6 Pharmacokinetic modelling

Therapeutic drug monitoring in conjunction with pharmacokinetic modelling can potentially improve the outcome of chemotherapy. Relationships between therapeutic or toxic effects and pharmacokinetic variables first need to be identified. The use of classical pharmacokinetic studies with large numbers of patients has significant logistical difficulties, as the collection of frequent multiple blood samples is required. This can be overcome by population pharmacokinetic modelling and maximum a posteriori probability (MAP) Bayesian estimation, which enables pharmacokinetic parameters to be defined using a small number of blood samples, with flexible sampling times. Bayesian pharmacokinetic forecasting takes population values and integrates patient specific data to determine individual doses.

Bayes theorem states that "prior beliefs, in the form of a probability distribution, may be combined with observed data, in the form of a likelihood function, to yield a posterior distribution" (Abrahms et al 1994). It leads to the following:-

Posterior probability $\propto$ likelihood * prior probability 6.12

Bayesian estimation firstly assesses the pharmacokinetic characteristics of a typical population by calculating the means and standard deviations of the pharmacokinetic parameters representing all patients included thereafter in a given study. To estimate individual pharmacokinetic parameter values, the Bayesian estimator then combines the prior knowledge of the probability density function of the population parameters with the fractional individual patient data, and possibly physiological parameters such as body weight, age or gender.

The predictive performance of the estimator must be evaluated. A second group of patients with similar characteristics to the individuals included in the reference population must be studied. The rich data (eg multiple blood concentrations) observed in each patient in this validation group, and more precisely the individual pharmacokinetic parameter values or variables derived from these data using another

fitting method (eg non-linear regression), need to be compared with those predicted by the MAP Bayesian estimator using only one or two concentrations.

One or two stage methods are used. In a two stage approach, the first stage estimates the pharmacokinetic parameter values of each patient using the pharmacokinetic model that best fits the concentration time curves. In the second stage, the mean, standard deviation and covariance of pharmacokinetic parameters are calculated. An extension of this approach is the iterative two stage MAP Bayesian method (Jelliffe et al 1998), where the individual pharmacokinetic parameter values are estimated using MAP Bayesian estimation, permitting the inclusion of patients for whom there is only one blood sample. The population model parameters are provided initially by a reasonable estimation determined in a very small number of patients. This population model is further combined with individual data to obtain the maximum a posteriori probability of the main pharmacokinetic parameter values of each patient using the Bayesian approach. The mean and standard deviation values of the parameters may be computed iteratively, the newly obtained mean and standard deviations being used as Bayesian priors for the following step. This iterative process ends when each of the sample mean values becomes stable.

One stage methods consider the data observed in all patients, to which the pharmacokinetic model is fitted. This provides information about which patient characteristics are important in determining the pharmacokinetics of the drug and how much variability can be accounted for by such characteristics. It is not necessary to obtain all the samples required to establish individual pharmacokinetic profiles, as these are not determined, and it is possible to scatter different sampling times between patients to the extent that each patient only needs to be sampled once. The most widely used model of the parametric one stage methods is the nonlinear mixed effect model. Each pharmacokinetic parameter is considered as the sum of the "fixed effect", provided by the population, and the "random effect", which depends on the individual. The most popular software utilising this approach is NONMEM (nonlinear mixed effect modelling). NONMEM estimates population parameters as

typical parameter values with the estimated interindividual variability usually as the standard deviation.

To date, therapeutic drug monitoring has only proved useful for a few anticancer drugs such as high dose methotrexate, 5-fluorouracil and the platinum compounds, due to the lack of identifiable relationships between response and systemic exposure (Rousseau et al 2000). Many more relationships have been demonstrated between exposure and toxicity. Kerbusch et al (2001) recently used the NONMEM programme to develop and validate a model to estimate ifosfamide, 2-DCEI, 3-DCEI, and 4-hydroxyifosfamide concentrations in a paediatric population using limited sampling. Cross-validation indicated no bias, and only minor imprecision ($12.5 \pm 5.1\%$) for 4-hydroxyifosfamide.

An improved knowledge of the relationships between the pharmacokinetic parameter values or exposure variables of cytotoxics and tumour growth or survival rate is needed. This could contribute to increased treatment efficacy and patient outcome by allowing an a priori determination of the first dose and a posteriori adjustment of the following doses.

Chapter 7

The Separation of Red Cells from Plasma and Measurement of Red Cell Volume

7.1 Introduction

Two approaches are usually used to determine the amount of a compound associated with red cells (chapter 4). In the first, the erythrocytes are washed prior to analysis, but this may strip them of their outer layer, which may be important in the transport and exchange process. A second technique is to measure the concentration in whole blood and subtract from this the amount present in plasma, corrected for the haematocrit. However, when a compound is found in high concentrations in plasma relative to red cells, this approach can be very inaccurate, and may even result in negative values when the erythrocyte associated concentration is calculated.

Ideally, a direct determination of red cell associated compounds should be performed on unwashed erythrocytes, maintained in their natural environment, without disruption of the normal equilibrium existing between red cells and plasma in whole blood. Before a direct analysis, the red cells must be compressed, reducing the amount of plasma between the cells to a fixed volume, as low as possible, approximately 2% (International Committee for Standardisation in Haematology 1980; Pearson and Guthrie 1982; Rustad 1964). The red cells need to be collected in a reproducible manner, and the volume of the collected cell mass must be known. The direct method employed by Driessen et al (1989a; 1989b; 1989c; 1990) is so inconvenient that it is not acceptable for routine use. Only one other example exists in the literature where a similar procedure was followed (Maling et al 1989).

If the plasma volume between red cells can be diminished, a more direct analysis of compounds associated with red cells can be performed. A sediment of red cells

obtained at 10,000 g will contain no more than 2% (v/v) of trapped plasma. It is therefore possible to separate off and measure such a cell sediment, containing a constant volume of trapped plasma, with the concentration of compounds on the red cells unchanged by the procedure. Thereafter, compounds associated with a known mass of red cells can be quantified. The MESED (measurement of sediment) instrument (FABRE, Kelmis, Belgium) allows such a separation, and was used for the measurement of red cell volume, prior to the analysis of ifosfamide and its metabolites in the plasma and red cell compartments.

7.2 The use of the MESED

7.2.1 The MESED instrument

A reservoir A is inserted into a container B, as shown in Figure 7.1. The reservoir is fitted tightly into the container so that the small orifice in the reservoir [1] is sealed by the o-ring in the container [2] (panel **a**). Fresh blood is collected and anticoagulated in lithium heparin vacutainers, the reservoir filled with an aliquot of blood, and the unit placed in a swing out rotor. By centrifuging at high speed (> 5000 g) red cells are forced into the narrow part of the reservoir [3], into the closed small channel [4], and into the bottom of the broad part of the reservoir [5]. After the first centrifugation some supernatant plasma is removed for independent analysis.

Before the second centrifugation, the reservoir A is detached by turning A in B, so that the o-ring [2] no longer occludes orifice [1] (panel **b**). During the second centrifugation, the flow resistant red cell sludge, together with the remaining supernatant plasma, is forced out of the reservoir A into the bottom of the container B, leaving behind a defined volume of almost pure red cells in the narrow part of A [3].

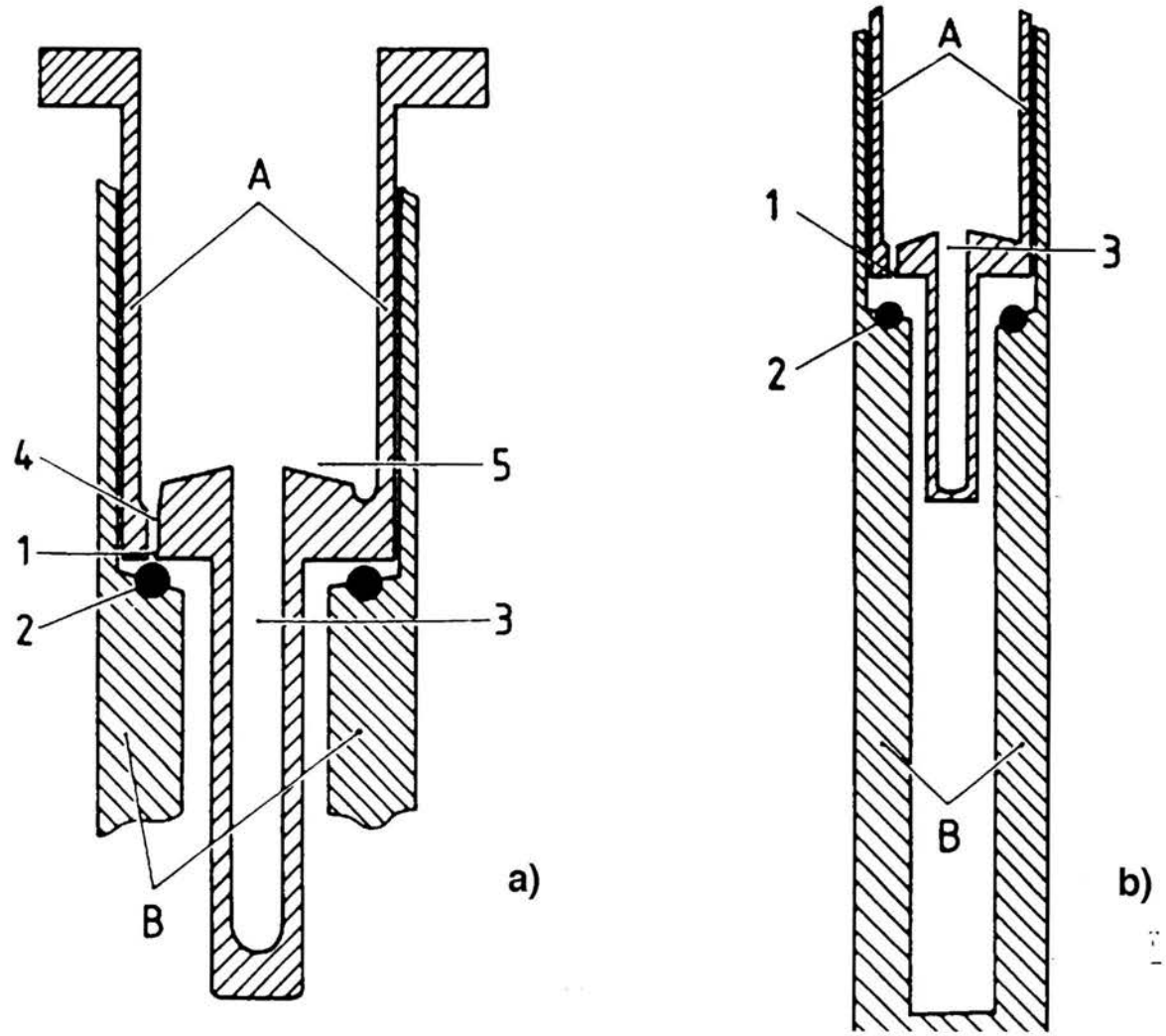


Figure 7.1. The MESED instrument.

This mass of cells is then transferred to a centrifuge tube by centrifuging the inverted reservoir alone. The weight of collected cells depends on the exact dimensions of the narrow part of the reservoir, on the centrifugal force applied, and on the density of the cells, the density of normal erythrocytes being 1.096.

A correction must be made for the plasma trapped in the sediment. The amount of trapped plasma was determined initially under standardised conditions using an extracellullar marker, ^{51}Cr-EDTA. The collection of supernatent plasma after the first centrifuge run, and its subsequent analysis, is required for this correction.

A mistral 3000 centrifuge was used (MSE Scientific Instruments, Crawley, UK), with plastic centrifuge tubes (Sarstedt Ltd, Leicester, UK) in the third centrifugation step. Centrifuge runs of 3600 g, 3600 g, and 1000 g, each for 10 minutes, were employed. At speeds greater than 1000 g in the third centrifugation, the centrifuge tubes fractured. This in turn limited the maximum speed in the first two centrifugations to 3600 g, as following higher speeds it was not possible to eject the sediment at 1000 g on the third centrifugation. The problem of sub-optimum speeds was overcome by weighing the centrifuge tubes before and after the third centrifugation, and calculating the weight of the erythrocyte sediment.

7.2.2 Calibration

Whole blood samples (1 ml) were spiked with 20 µl of ^{51}Cr-EDTA, (3 MBq in 10 ml) (table A1, appendix). The blood samples were mixed well at room temperature, allowed to equilibrate for 15 minutes, mixed well again, and plasma and erythrocytes separated using the MESED tube, as described above. 100 µl of plasma was used for the γ count, and the red cells were suspended in 150 µl of 0.9% saline before counting. The calibration samples were counted in a 1282 compugamma CS Universal gamma counter (LKB Wallac, Perkin Elmer, Denmark). The median percentage of trapped plasma in the erythrocyte sediment was 3.72%. This is somewhat higher than that reported by Siebers and colleagues, and may reflect differences in the behaviour of

^{51}Cr-EDTA and (Co^{++})-EDTA (Brochner-Mortensen et al 1969; Burck 1970; Siebers et al 1986).

7.3 Assay of ifosfamide and its metabolites in erythrocytes

The red cell sediments generated by the MESED tube were haemolysed by adding 0.9 ml of HPLC quality water, vortexing, and leaving the mixture at room temperature for 30 minutes. The lysate was diluted with 1 ml of buffer, and then treated as plasma samples in the subsequent extraction process (chapter 5). Species adsorbed or incorporated into the red cell are not distinguished, and the erythrocyte concentrations are minimal values. Although it is not possible to exclude an incomplete recovery of active compounds from red blood cells, recovery was not increased with repeated extraction of the erythrocyte lysate. Erythrocyte concentrations (E) were determined using the calibration curve obtained for spiked plasma (500 µl):-

$$E = P_{cal} \times 500/\text{weight of erythrocytes (mg)} \qquad 7.1$$

where P_{cal} is the reading obtained from the spiked plasma calibration curve.

E was expressed in mg Kg^{-1} of red blood cells, or µg ml^{-1}, assuming an erythrocyte density of 1.

A correction was made for the amount of trapped plasma (3.72%):-

$$E_c = E + (E \times 0.0372) - (P \times 0.0372) \qquad 7.2$$

Where E_c is the corrected red cell concentration, E is the measured erythrocyte concentration and P is the plasma concentration.

7.4 Conclusion

The MESED instrument allows a direct analysis of the erythrocyte compartment, without disturbing the equilibrium present between erythrocytes and plasma. Simultaneous analysis of plasma can also be performed.

PART III RESULTS

Chapter 8

The Assessment of Neurotoxicity

8.1 Introduction

Ifosfamide induced neurotoxicity can be assessed using the EEG, psychometric testing, the national adult reading test (NART), the mini-mental state (MMS) examination, and clinical evaluation of symptoms.

8.1.1 The electroencephalogram

The EEG is the electrical activity recorded by conventionally placed electrodes over the scalp. It is an attenuated and smoothed form of the electrocorticogram. In the wakened but relaxed state, rhythmical sinusoidal potentials wax and wane. A characteristic of the human EEG is the 8 Hz to 12 Hz alpha rhythm in areas posterior to the central sulcus, occurring independently in both hemispheres, but with similar frequency on each side. The waves are not action potentials, but are believed to represent synchronised dendritic potentials superimposed on slowly varying potential differences across the cortex (DC shifts). Arousal, and especially fixation of attention on a visual pattern, or performance of mental activity such as calculation, desynchronizes the alpha rhythm. The synchronised state of the EEG, in the wakened but relaxed state, results from the inhibitory effect of thalamocortical fibres on the higher frequency driving of cortical synapses affected by the mesencephalic reticular formation. When the latter dominates during alerting, the alpha rhythm is desynchronized. The transition from wakefulness to drowsiness is marked by increased amplitude of post-central alpha rhythm, and its frequency may slow by 0.5 Hz to 1.0 Hz.

Several traditional frequency bands have been used as measures of drug effects (table

8.1). Although there is physiological evidence for the independence of these frequency bands, their boundaries are not strict, and may differ depending on species, population and experimental conditions (Mandema and Danhof 1992).

Band	Hz
alpha	8 – 14
alpha 1	8 – 10
alpha 2	10 – 14
beta	14 – 32
beta 1	14 – 20
beta 2	20 – 32
delta	0 – 4
theta	4 – 8

Table 8.1. Frequency of EEG band widths.

The introduction of computers has facilitated the quantification and processing of drug induced EEG changes. Quantitative EEG analysis is used now in many areas of clinical pharmacological research, and can provide information on the intrinsic effects of centrally acting drugs. Different classes of such drugs produce characteristic EEG changes. Quantitative EEG analysis can in principle be used to characterise pharmacokinetic – pharmacodynamic (PK–PD) relationships. EEG parameters appear to possess several of the ideal features for pharmacodynamic measurements; continuity, objectivity, sensitivity and reproducibility.

8.1.2 Psychometric tests

The aim of most behavioural or performance research in relation to sedative compounds is to determine if their use causes a diminished ability to perform routine, complex psychomotor tasks. The most common example of such a task is driving a car.

a Reaction time

These tests evaluate motor response by requiring the subject to press a button in response to a critical stimulus. The number correct, and the latency to respond, are normally used as a measure of task performance. Both simple and choice reaction time have been employed. Simple reaction time involves only a motor response, for example, how rapidly the subject presses a button after stimulus presentation. In choice reaction time, the subject is presented with a single stimulus that is one of a number of alternatives. This test assesses sensorimotor performance by adding a recognition time component (stimulus processing time) to the motor movement aspect of the simple reaction time test.

b Digit symbol substitution

This has been presented in a number of different forms. The task requires sustained attention and concentration, and evaluates response speed and recognition of sensory information.

c Tracking

Tracking is an assessment of visuo-motor co-ordination, and may contain elements of reaction time, fine and course motor control, and attention. The pursuit rota is regarded as the most basic measure of visuo-motor performance. Compensatory and adaptive tracking use the input of the subject while attempting to maintain the position of an indicator on screen. The root mean square of the distance of the indicator from the centre of the screen is usually the performance measurement in these tasks. Pursuit tracking requires the subject to follow a path generated by the device and superimpose their own path.

The types of test that may be included in a testing battery are many and varied, but the most commonly applied tests are compensatory tracking, and simple and choice reaction time tests.

8.1.3 National adult reading test

The NART is a universally applicable indicator of premorbid intelligence levels. Reading ability is highly correlated with general IQ level in the normal population, and the NART was specifically designed to provide a means of estimating the premorbid intelligence levels of adult patients suspected of suffering from intellectual deterioration.

8.1.4 Mini-mental state examination

The MMS examination is a quick and valuable test for simple bedside screening and serial assessment of cognitive function (Dick et al 1984).

8.2 Methods

Eleven patients receiving oral ifosfamide 500 mg twice daily were monitored for neurotoxicity on their first cycle of treatment. Ifosfamide was administered for a period of 14 days, and assessments were performed on days 0 (baseline), 2, 8 and 15. Cycles were repeated every 28 days. Seven patients had cervical carcinoma, and four NSCLC.

8.2.1 Electroencephalogram system

The international 10/20 system of EEG electrode placement was used. Twenty-one channels were recorded through a bandwidth of 0.5 Hz to 30 Hz. The standard sequence

was 20 seconds eyes open, 20 seconds eyes closed. Epochs of eight seconds were taken for analysis commencing five seconds after eye opening or closure. Procedures were continued until ten artefact-free epochs were obtained under both eyes open and eyes closed conditions.

The EEGs were evaluated blindly and independently by a neurologist, with only the patient initials given. The day of investigation and whether the patient was receiving treatment was not indicated. Two main measures were employed. The first was a system of evaluating the basic background alpha rhythm at 27 different one second epochs. The mean alpha rhythm of each recording was then determined. The second evaluation of the recordings assessed the EEG abnormalities, particularly slow activity, episodic slow activity and paroxysmal activity. In addition, the changes in the recording due to over breathing and photic stimulation were evaluated. The results were listed and the records given an arbitrary ranking from the most normal to the most abnormal, according to the Meanwell grading (table 3.3). The code was then broken, and the rankings compared to the actual days of the study, 0 (baseline), 2, 8 and 15.

8.2.2 Psychometric tests

The psychometric system used in the Guy's drug research unit was developed to provide a comprehensive set of fully automated procedures for evaluating the psychological and behavioural consequences of compounds with potential CNS activity. The system comprises a battery of psychometric tests installed on IBM microcomputers, which are fully automated and require the minimum of supervision. The tests consist of choice reaction time, a continuous attention task, and the salford tracker, which are considered to be amongst the most sensitive tests available for psychopharmacological effects. Psychometric tests were performed in the following order: choice reaction time, salford tracker and continuous attention task.

a Choice reaction time

This comprises two parts; recognition and motor reaction times (table 8.2). Five practice tests are allowed, followed by 20 live tests.

Test	Description
CR 1	Mean time to take pen off (reaction time)
CR 2	SD of time to take pen off
CR 3	Mean time to put pen on
CR 4	SD of time to put pen on

Motor response time is CR 3 – CR 1

Table 8.2. Choice reaction.

b Salford tracker

This test is a simulated car driving test in which the subject tracks an arrow using a computer mouse. The maximum distance from the target and the root mean square of this distance is recorded (table 8.3). In addition, the subject responds to a stimulus presented in the periphery of vision while simultaneously attending to the tracking task. The maximum and average response times to ten of these peripheral stimuli per task are also used as response measures. The distance is measured in pixels, and when superimposition is perfect the score is zero. One practice session is permitted before three live sessions.

c Continuous attention task

In this test, patients monitor a series of digits for targets of three consecutive odd or three consecutive even digits, and signal their detection by pressing a response button as

quickly as possible. The outcome measures from this task are the average reaction time to respond to the targets, the number of missed responses, and the number of false responses. Two hundred numbers are presented, and each number is displayed for one second.

Test	Description
ST 1	Mean minimum time
ST 2	SD of minimum time
ST 3	Mean maximum time
ST 4	SD of maximum time
ST 5	Mean time (to press button)
ST 6	SD of time (to press button)
ST 7	Mean maximum distance ("veering" distance with driving test)
ST 8	SD of maximum distance
ST 9	Mean average distance (from the point of perfect superimposition)
ST 10	SD of average distance

ST 1 to ST 6 is to stimulus whilst tracking

Table 8.3. Salford tracker.

Test	Description
CA 1	Mean response time
CA 2	SD response time
CA 3	Number of correct sequences missed
CA 4	Number of false responses

16 correct sequences were present in the test

Table 8.4. Continuous attention task.

8.2.3 National adult reading test

The NART comprises a list of 50 words printed in order of increasing difficulty (tables 8.5 a-c). They are irregular with respect to the common rules of pronunciation to minimise the possibility of reading by phonemic decoding rather than word recognition.

WORD	PRONUNCIATION GUIDE	CORRECT	INCORRECT
CHORD	körd		
ACHE	āk		
DEPOT	dep ' ō		
AISLE	il		
BOUQUET	bōōk ' ā, bōōkā, bōkā '		
PSALM	säm		
CAPON	kā ' pn		
DENY	di-ni		
NAUSEA	nö ' si-a, nö ' zha		
DEBT	det		
COURTEOUS	kûrt ' yas		
RAREFY	rār ' -i-fi		
EQUIVOCAL	i-kwiv ' a-kl		
NAÏVE	nä-èv		
CATACOMB	kat ' a-kōōm		
GAOLED	jāld		

Table 8.5 a. Word lists; only the left hand column was given to the patient.

WORD	PRONUNCIATION GUIDE	CORRECT	INCORRECT
THYME	tim		
HEIR	ār		
RADIX	rā ' diks		
ASSIGNATE	as ' -ig-nāt		
HIATUS	hi-ā ' tas		
SUBTLE	sut ' l		
PROCREATE	prō ' kri-āt		
GIST	jist		
GOUGE	gowj		
SUPERFLUOUS	sȱō-pûr ' flōō-as, sū-pûr ' flōō-as		
SIMILE	sim ' l-li		
BANAL	ban-al '		
QUADRUPED	kwod ' rōō-ped		
CELLIST	chel ' ist		
FACADE	fa-säd '		
ZEALOT	zel ' at		
DRACHM	dram		

Table 8.5 b. Word lists; only the left hand column was given to the patient.

WORD	PRONUNCIATION GUIDE	CORRECT	INCORRECT
AEON	ē ' on		
PLACEBO	pla-sē ' bō		
ABSTEMIOUS	ab-stē ' mi-as		
DÉTENTE	dā-tāt (Fr)		
IDYLL	id ' il, id ' al		
PUERPERAL	pū-ûr ' par-al		
AVER	a-vûr '		
GAUCHE	gō sh		
TOPIARY	tō ' pi-a-ri		
LEVIATHAN	le-vì ' a-than		
BEATIFY	bi-at ' i-fī		
PRELATE	prel ' it		
SIDEREAL	si-dē ' ri-al		
DEMENSE	di-mān ', di-mēn '		
SYNCOPE	sing ' ka-pē		
LABILE	lā ' bil		
CAMPANILE	kam-pan-ē ' lā, kam-pan-ē ' lē		

Table 8.5 c. Word lists; only the left hand column was given to the patient.

The patient reads aloud down the word lists, and the examiner records the number of errors made according to the pronunciation guide. The Wechsler adult intelligence scale (WAIS) full-scale, verbal, and performance IQs were predicted from this reading error score by inserting it into the appropriate formula (Wechsler 1955):-

WAIS full scale IQ = 127.7 – (0.826 x NART error score)

WAIS verbal IQ = 129.0 – (0.919 x NART error score)

WAIS performance IQ = 123.5 – (0.645 x NART error score)

8.2.4 Mini-mental state examination

The MMS test is shown in table 8.6.

8.2.5 Clinical assessment

Neurotoxicity was also assessed clinically. Patients were graded according to the common toxicity criteria (table 3.2).

8.2.6 Probability of encephalopathy

The probability of developing encephalopathy was calculated according to the method of Meanwell et al (1986a). Weights for serum albumin, serum creatinine and the presence of pelvic disease, together with constants derived from the analysis, were used to determine classification scores (S1 and S2) for each patient.

S1 = -31.3381 + (1.1650 x [albumin]) + (0.1527 x [creatinine]) + (5.2841 x [pelvic value])

Question	Points	Question	Points
ORIENTATION Time (5)	_____	LANGUAGE 1 point for 2 objects correctly named (2)	_____
Place (3)	_____		
REGISTRATION Name 3 objects (3)	_____	1 point if the following sentence is repeated correctly NO IFS AND BUTS (1)	_____
ATTENTION & CALCULATION Subtract 7 from 100 and 7 from the result. (Repeat 5 times) (5)	_____	3 points if a 3-stage command is correctly executed (3) Write "close your eyes" and ask subject to obey what is written - 1 point if subject closes eyes (1)	_____ _____
RECALL Ask for 3 objects repeated in the REGISTRATION test (3)	_____	Subject writes sentence; 1 point if sensible, with verb and subject (1) Construct a pair of pentagons 1 inch long; 1 point if subject copies correctly (1)	_____ _____
Sub Total: (19)	_____	+ Sub Total: (9)	_____
TOTAL SCORE (max 28) =	_____		

Table 8.6. Mini-mental state assessment.

$S2 = -28.5367 + (0.8398 \times [albumin]) + (0.2055 \times [creatinine]) + (7.1589 \times [pelvic\ value])$

The pelvic value for patients with and without a pelvic lesion is 2 and 1 respectively. Albumin is measured in g L^{-1} and creatinine in μM.

The probability of a patient developing grade 0 to 2, rather than grade 3 or 4, encephalopathy according to the Meanwell grading is $e^{S1}/(e^{S1}+e^{S2})$.

8.3 Results

8.3.1 Electroencephalogram

Seven patients were assessed. In all except one, the EEG was abnormal by day 2, with decreased alpha frequency and the development of pathological slow wave activity. The changes in the recordings were quite dramatic, and showed some of the features seen in toxic encephalopathies, and even changes observed in patients with epileptigenic disturbances. An example of an EEG on day 2 is given in figure 8.1. The results in some of the patients showed a perfect ranking order. In others this was not perfect, but there was always an isolation of the records of the 8th and 15th days from those of the baseline and the 2nd day (table 8.7).

8.3.2 Psychometric tests

Six patients were subjected to psychometric testing. The psychometric data during the first course of oral ifosfamide are shown in tables A2 to A4, and graphic representations illustrated in figures 8.2 to 8.4. The mean reaction time and the mean time to place the pen in the choice reaction test was impaired in all patients except one, although this failed to reach statistical significance (table A5).

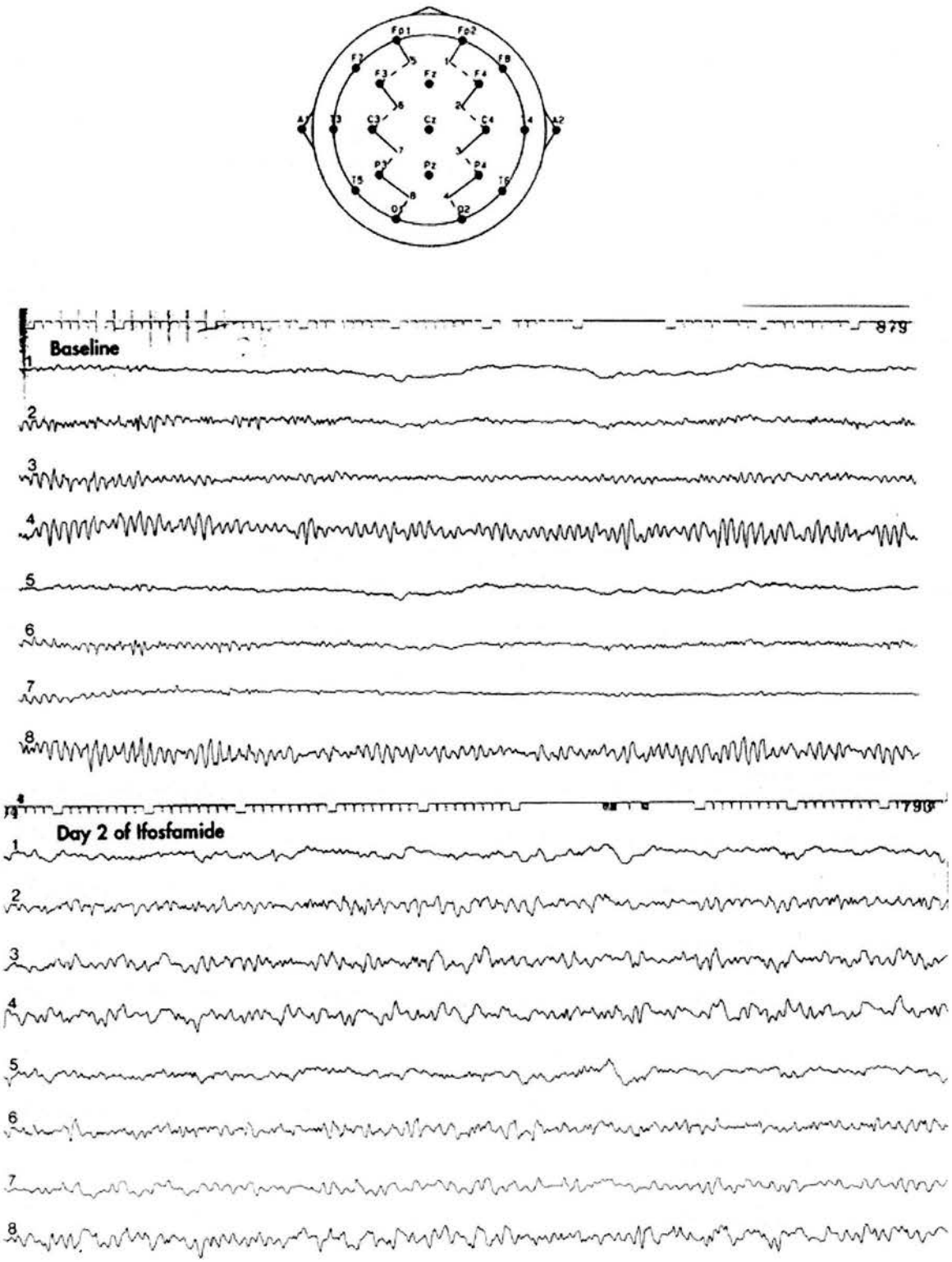


Figure 8.1. EEGs on day 0 (baseline) and day 2 in patient SO.

The mean average distance in the salford tracker was significantly increased on day 15 ($p = 0.026$). The mean response time in the continuous attention task was significantly increased on day 2 ($p = 0.018$).

Patient	EEG score				Mean alpha frequency (Hz)					
	Day				Day					
	0	2	8	15	0	2	% change 0 to 2	8	15	% change 0 to 15
CB	0	0	-	-	9.48	9.86	104.0	-	-	-
EG	0	2	2	3	10.90	9.92	91.0	8.90	8.52	78.2
CH	0	1	3	3	9.68	8.54	88.2	7.62	8.00	82.6
JW	0	1	2	2	9.78	9.54	97.5	8.92	8.46	86.5
LR	0	2	-	3	10.54	8.48	80.5	-	6.98	66.2
SO	0	1	-	-	9.94	7.70	77.5	-	-	-
NH	0	3	-	-	10.42	-	-	-	7.35	70.5

EEG grading is according to Meanwell et al (1986a)
Rank correlation, day against percentage change in alpha frequency, is 0.753; $p < 0.001$

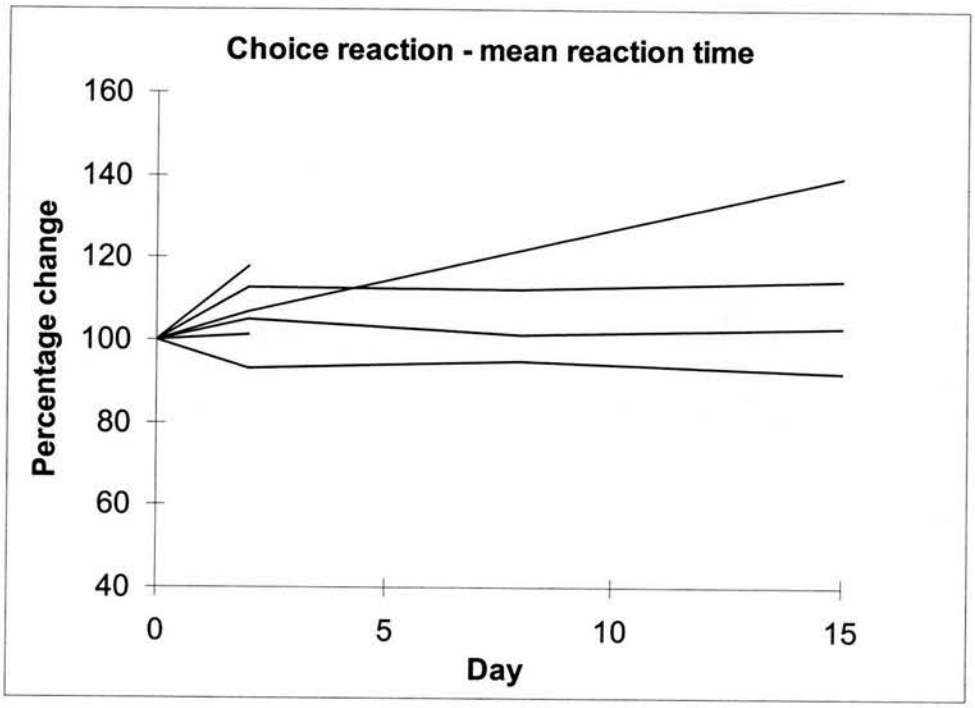
Table 8.7. Changes in EEG score and mean alpha frequency during oral ifosfamide.

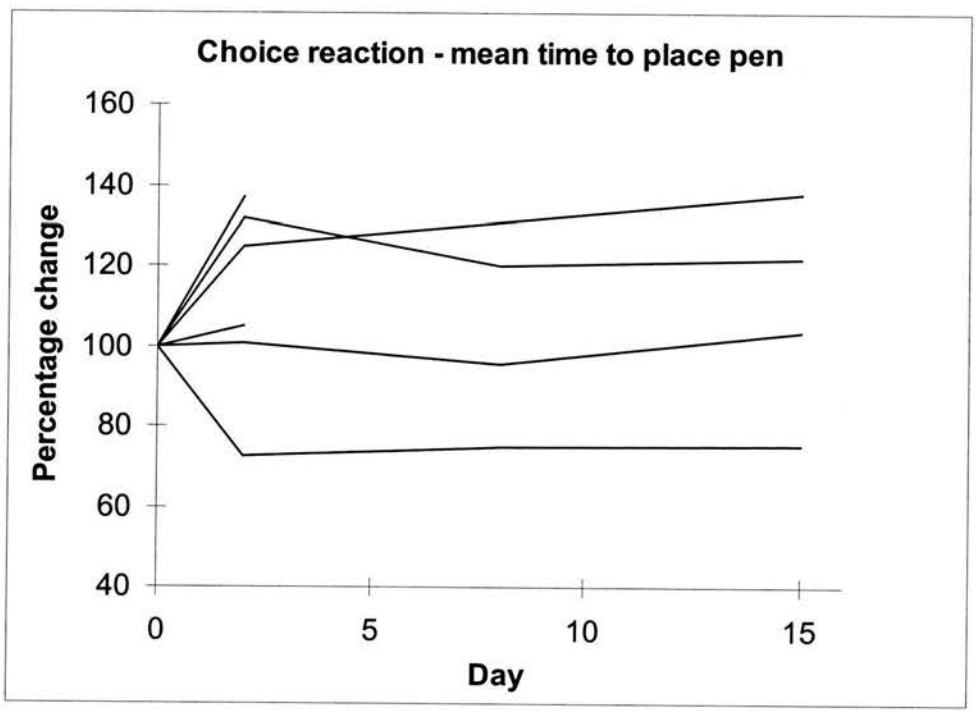
8.3.3 National adult reading test

The results of the NART are given in tables 8.8 and 8.9. Performance decreased slightly on day 2 in two patients, but was otherwise unchanged.

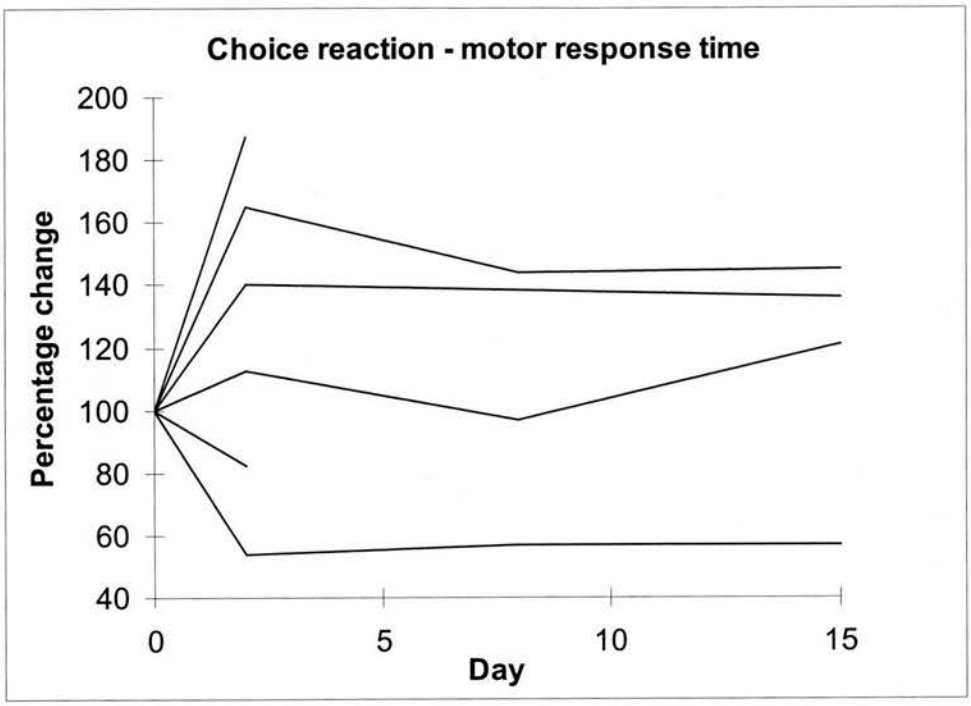
8.3.4 Mini-mental state examination

No significant changes in this score were observed during oral ifosfamide administration (table 8.10).





Figures 8.2 a and b. Choice reaction time.



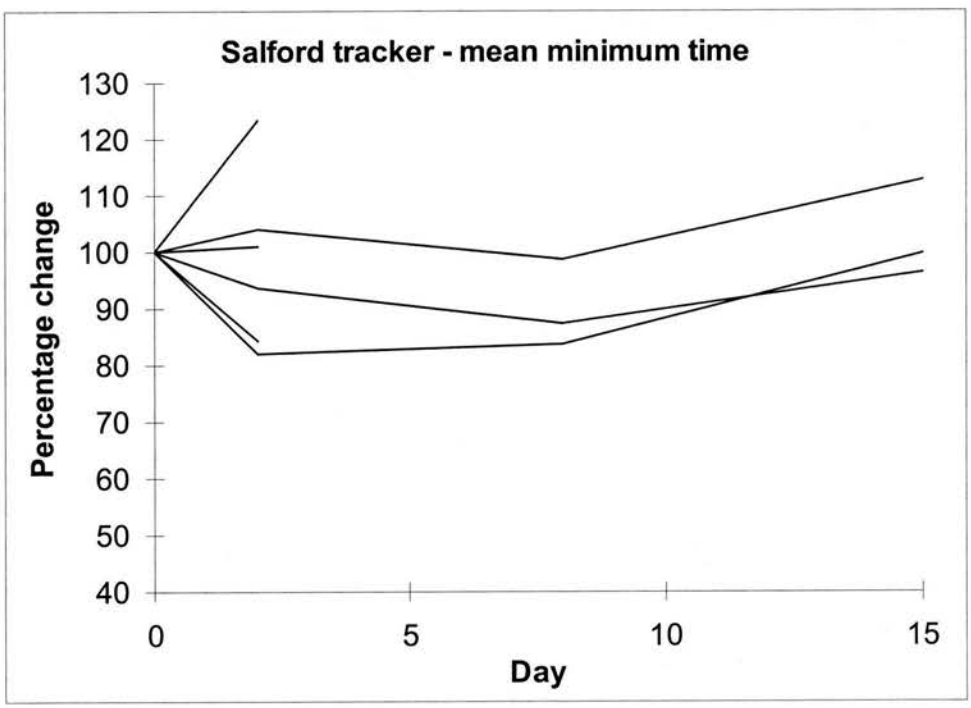
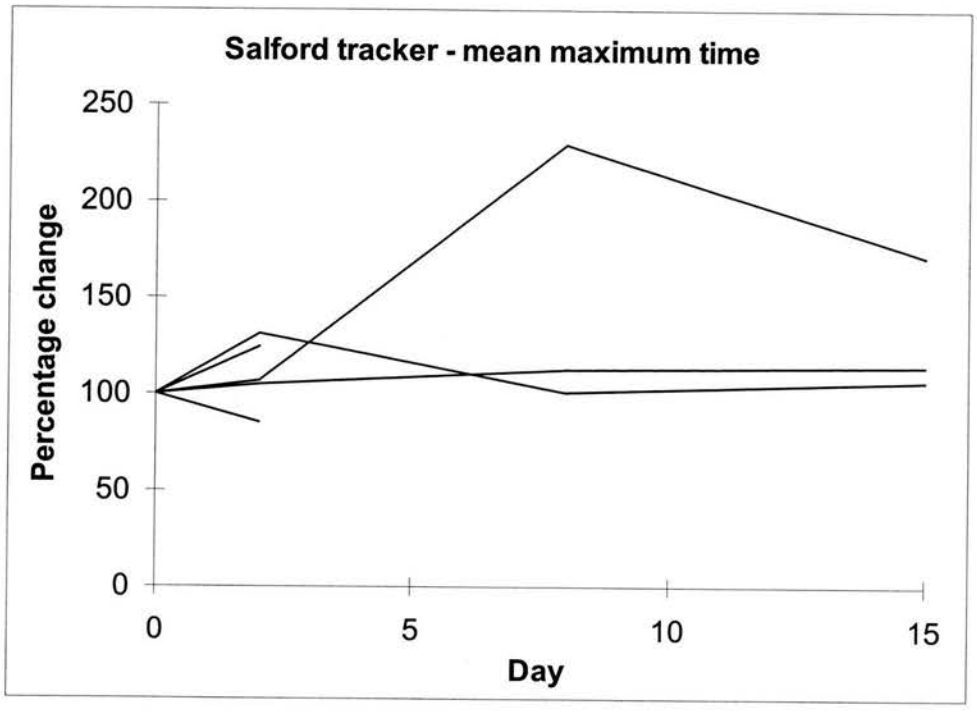
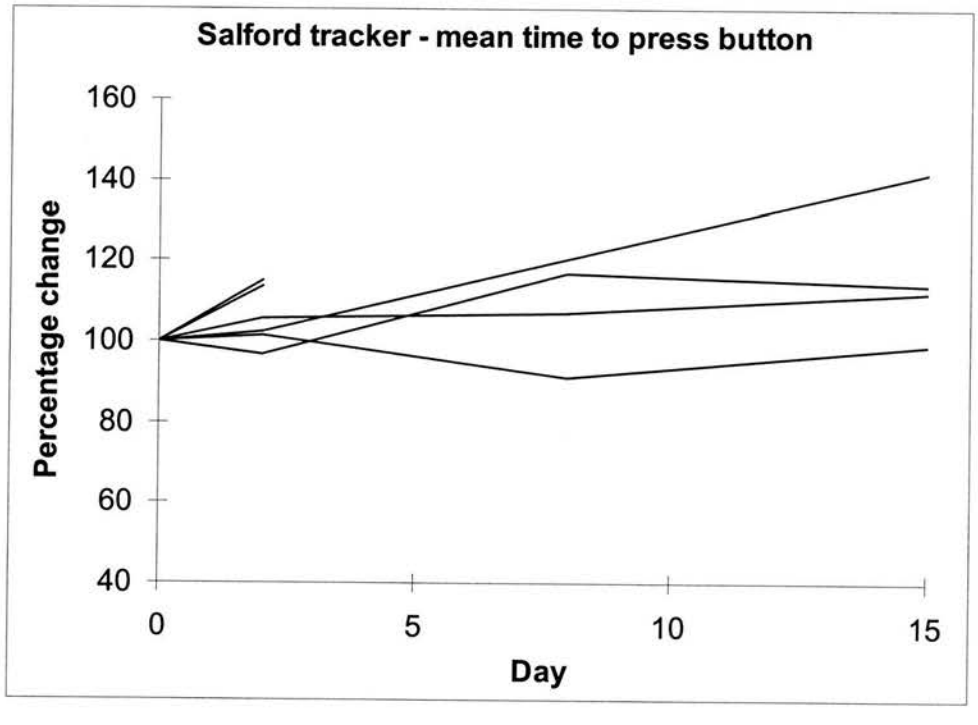


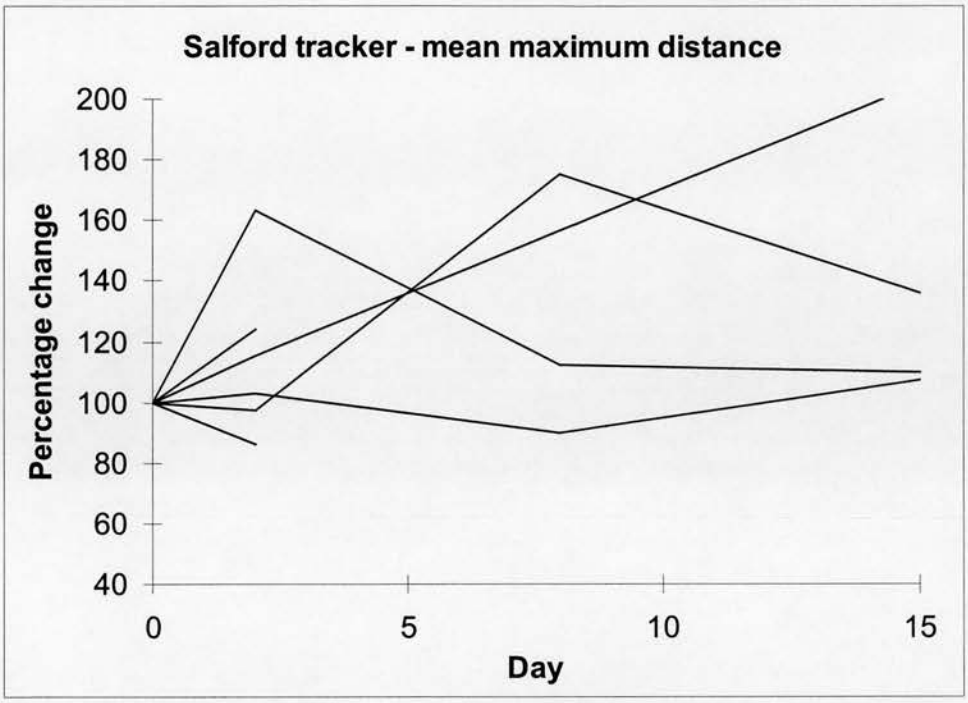
Figure 8.2 c (upper). Choice reaction time.

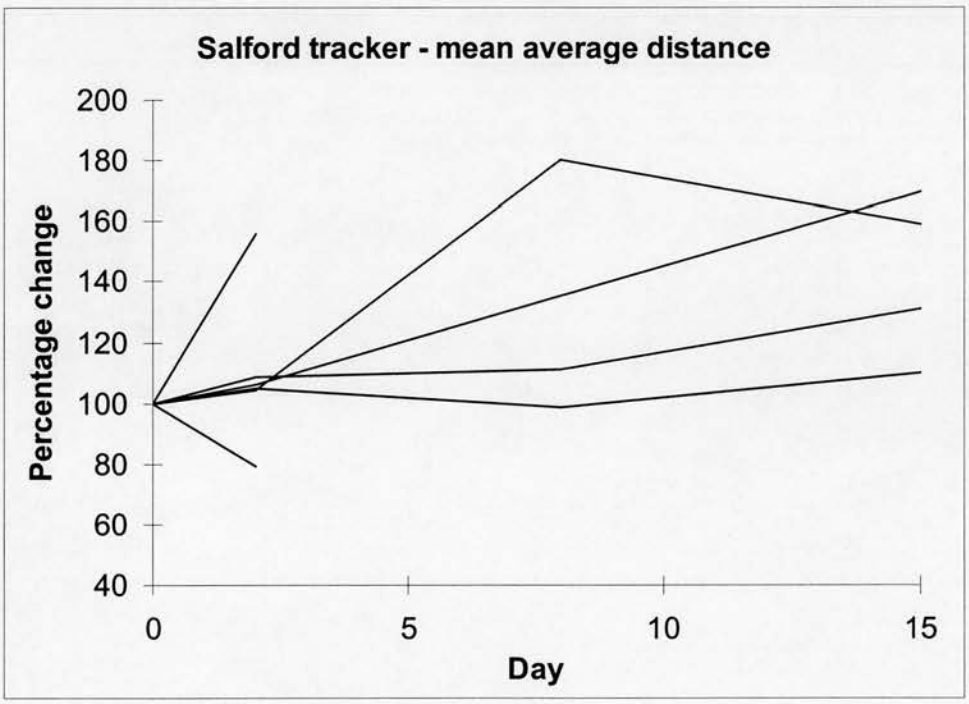
Figure 8.3 a (lower). Salford tracker.





Figures 8.3 b and c. Salford tracker.





Figures 8.3 d and f. Salford tracker.

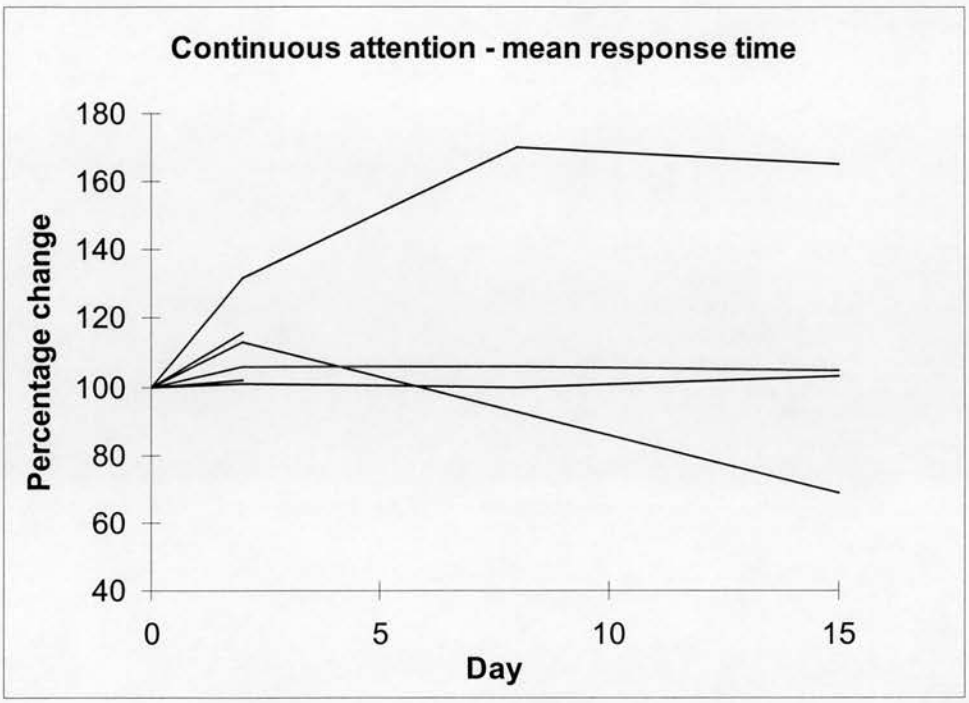


Figure 8.4. Continuous attention task.

8.3.5 Clinical assessment

The neurotoxic symptoms experienced by the patients are described in table 8.11. Two patients are not assessable for neurotoxicity, one dying suddenly from a pulmonary embolus on the fourth day of oral ifosfamide. All assessable patients developed a feeling of detachment, or an inability to concentrate. In addition, four patients suffered neurocortical disturbances and one anxiety. Neurological manifestations were evident by day 3 of treatment in all patients, and resolved on discontinuation of ifosfamide. The Meanwell grading of the EEGs (table 3.3) was not consistent with the clinical features; a score of 2 or 3 in five patients was associated with relatively mild neurotoxicity, whilst hallucinations occurred in one patient with a score of 1. However, two patients in the former group developed severe neurotoxicity with subsequent courses.

Patient	Day 0		Day 2		Day 8		Day 15	
	C	W	C	W	C	W	C	W
CB	37	13	45	5	-	-	-	-
EG	45	5	44	6	45	5	45	5
CH	32	18	42	8	40	10	34	16
JW	20	30	25	25	27	23	25	25
LR	41	9	46	4	-	-	42	8
SO	26	24	23	27	-	-	-	-

C = Number correct; W = Number wrong

Table 8.8. National adult reading test.

Patient	Day 0			Day 2			Day 8			Day 15		
	F S	V	P	F S	V	P	F S	V	P	F S	V	P
CB	117	117	115	124	124	120	-	-	-	-	-	-
EG	124	124	120	123	123	120	124	124	120	124	124	120
CH	113	112	112	121	122	118	119	120	117	114	114	113
JW	103	101	104	107	106	107	109	108	109	107	106	107
LR	120	121	118	124	125	121	-	-	-	121	122	118
SO	108	107	108	105	104	106	-	-	-	-	-	-

F S = Full scale IQ; V = Verbal IQ; P = Performance IQ

Table 8.9. National adult reading test; predicted IQ.

Patient	Day 0	Day 2	Day 8	Day 15
CB	26	28	-	-
EG	-	26	25	25
CH	28	25	26	26
JW	25	28	28	27
LR	28	28	-	28
SO	21	22	-	-

Table 8.10. Mini-mental state examination score.

Patient	PD	Course 1			
		Albumin ($g\ L^{-1}$)	Creatinine (μM)	Symptoms	CTC (grade)
CB	No	31	82	Not assessable	
EG	Yes	44	113	Mildly impaired concentration	
CH	Yes	45	114	Mild feeling of detachment	
JW	Yes	49	89	Mildly impaired concentration	
LR	Yes	42	94	Anxiety	Neuromood (1)
SO	Yes	33	62	Disorientation + hallucinations	Neurocortical (3)
PC	No	35	80	Somnolence	Neurocortical (2)
RC	No	34	123	Hallucinations	Neurocortical (3)
JT	No	33	88	Mildly impaired concentration	
NH	No	42	69	Somnolence	Neurocortical (1)
LJ	Yes	39	107	Not assessable	

PD = Pelvic disease

Table 8.11. Clinical features during course 1 of oral ifosfamide.

8.3.6 Probability of encephalopathy

The probability of each patient remaining free from grade 3 or 4 encephalopathy is shown in table 8.12. The two patients with grade 3 CTC encephalopathy scored relatively low probabilities of 0.47 and 0.71.

Patient	Pelvic value	S1	S2	e^{S1}	e^{S2}	$e^{S1}+e^{S2}$	Probability
CB	1	22.6	21.5	6.42×10^{9}	2.19×10^{9}	8.61×10^{9}	0.75
EG	2	47.7	46.0	5.44×10^{20}	9.07×10^{19}	6.35×10^{20}	0.86
CH	2	49.1	47.0	2.03×10^{21}	2.58×10^{20}	2.29×10^{21}	0.89
JW	2	49.9	45.2	4.72×10^{21}	4.36×10^{19}	4.76×10^{21}	0.99
LR	2	42.5	40.4	2.91×10^{18}	3.41×10^{17}	3.25×10^{18}	0.90
SO	2	27.1	26.2	6.14×10^{11}	2.48×10^{11}	8.61×10^{11}	0.71
PC	1	26.9	24.5	5.00×10^{11}	4.18×10^{10}	5.41×10^{11}	0.92
RC	1	32.3	32.5	1.11×10^{14}	1.24×10^{14}	2.35×10^{14}	0.47
JT	1	25.8	24.4	1.65×10^{11}	4.03×10^{10}	2.05×10^{11}	0.80
NH	1	33.4	28.1	3.24×10^{14}	1.56×10^{12}	3.26×10^{14}	1.00
LJ	2	41.0	40.5	6.42×10^{17}	3.97×10^{17}	1.04×10^{18}	0.62

Table 8.12. The probability of developing grade 0 to 2, rather than grade 3 or 4, encephalopathy on course 1 of oral ifosfamide.

8.4 Discussion

Oral ifosfamide causes significant neurotoxic effects. All patients developed symptoms during the 14 days of treatment. A feeling of detachment, or an inability to concentrate, was universal, and one patient developed anxiety, two somnolence, and two suffered hallucinations.

Other studies have highlighted this type of toxicity. Four of six patients treated by Wagner and Drings (1986) experienced neurotoxicity after 2 g m^{-2} of oral ifosfamide, whilst an incidence of 33% was reported after daily doses of 2 g (Cerny et al 1989).

Doses lower than 1 g daily, or a shorter duration of treatment than 14 days, appear more tolerable. Manegold et al (1992) assessed four different schedules of oral ifosfamide (1 g daily for 5 days; 750 mg, 1 g or 1.25 g daily for 14 days [schedules 1 to 4 respectively]) in 64 patients with stage IV NSCLC. Neurotoxicity was measured according to Meanwell et al (1986a). Grade 1 or 2 CNS toxicity was seen in 2/15 (13%) of patients on schedule 1, 8/19 (42%) on schedule 2, 8/23 (35%) on schedule 3, and 4/7 (57%) on schedule 4. No grade 3 toxicity was seen. All episodes resolved on discontinuation of treatment. Somnolence and nightmares occurred most frequently, but hallucinations, restlessness and forgetfulness were also recorded. With mild to moderate encephalopathy ifosfamide was stopped. When rechallenged, five of 18 patients again developed encephalopathy. The time of onset of the encephalopathy was variable, but occurred in most patients on the second or third day. Neurotoxicity was evident in the first cycle in 15 cases. In the remaining seven cases neurotoxicity was first apparent in the second or subsequent cycles. The increase in the daily ifosfamide dose to 1.25 g led to increased neurotoxicity suggesting a relationship between dose and neurotoxicity. The total cycle dose was less important than the total daily dose.

Oral ifosfamide appears to have quite a prominent effect on cerebral function, at least as evaluated by the EEG, with decreased alpha frequency and the development of pathological slow wave activity. Other groups have also described a reduction in alpha frequency (Danesh et al 1989). Psychometric performance was impaired during the course of oral ifosfamide. This was particularly so for the mean response time in the continuous attention test, and the mean average distance in the salford tracker test. Cognitive function and IQ were not affected, although Heim et al (1981) have described impairment of short term memory in nephrectomised patients given intravenous ifosfamide.

Oral ifosfamide 500 mg twice daily for 14 days is too neurotoxic for routine use. The two patients with grade 3 CTC encephalopathy scored relatively low probabilities of remaining free from this degree of neurotoxicity using the Meanwell assessment,

indicating that this predictive approach may be useful for ifosfamide given orally. However more data is needed to develop precise discriminatory values.

Chapter 9

The Pharmacokinetics of Oral Ifosfamide

9.1 Introduction

Ifosfamide is usually administered intravenously. Daily fractionation of the dose over five days results in an increase in the Cmax and AUC of total alkylating activity, as measured by the NBP reaction (Lind et al 1989a). IPM, CIPA, DCEI, and ifosfamide at high concentrations, all have alkylating activity in this reaction (chapter 5). Therefore one would expect that fractionation would lead to an increase in cytotoxicity. Early work confirmed that this type of administration was effective, and further studies suggested reduced toxicity with five day regimens (Klein et al 1984; Morgan et al 1982; Rodriguez et al 1976). Fractionated ifosfamide therapy has become one of the most commonly used methods of administration. Routine clinical practice is to administer the drug in three or five day fractionated regimens, or as continuous infusions (Brade et al 1985). An oral fractionated regimen would be advantageous, minimising hospital admissions; an oral formulation, with a bioavailability of 100%, was described in 1986 (Wagner and Drings 1986), but its use has been restricted by an increased incidence of encephalopathy (Manegold et al 1992) (chapter 3).

Ifosfamide is a pro-drug, requiring activation within the hepatic cytochrome P-450 system to form IPM, the ultimate alkylating agent (chapter 2). Dechloroethylation, with the formation of chloroacetaldehyde, may be important in the development of encephalopathy. Other ifosfamide metabolites are theoretically possible, as illustrated by the metabolism of the structural isomer cyclophosphamide. This follows a similar metabolic fate, but NNM has also been found after intravenous administration in man. In one report the source of NNM was not clear as cyclophosphamide, 4-ketocyclophosphamide, carboxyphosphamide and CPM were all capable of

decomposing to NNM during the freezing and thawing of plasma (Jardine et al 1978). When CPM is injected into mice NNM is formed, and this in turn yields 3-(2-chloroethyl)-1, 3-oxazolidine-2-one (Struck et al 1975). In the case of IPM, an equivalent transformation leads to CEA and OXA (chapter 2); CEA has alkylating activity, but only one-fifth the potency of NNM (Sladek 1977).

This chapter is concerned with the pharmacokinetics of oral ifosfamide, and the generation of 2-DCEI, 3-DCEI, CIPA, KIPA, IPM, and the basic metabolites CEA and OXA, both of which have not been described previously in biological fluids.

9.2 Methods

Eleven patients were studied, seven females with advanced or recurrent carcinoma of the cervix, and four males with advanced NSCLC. The median age was 58 years (range 33 – 70). None had received prior chemotherapy. Oral ifosfamide, formulated as gelatin capsules, was administered at a dose of 500 mg twice daily, eight hours apart, for 14 days; film-coated tablets containing 300 mg of mesna were given twice daily concurrently for uroprotection.

Blood samples were taken before the first dose on days 1 and 14, and at approximately 0.5, 1, 2, 4 and 6 hours thereafter. The fasting state was maintained during the initial four hours. Plasma was separated immediately by centrifugation, and stored at -20°C until analysis. Ifosfamide and its metabolites were determined by GC–MS, as described in chapter 5. Pharmacokinetic data was obtained using the MK-MODEL programme (Holford 1990). The AUC was calculated using the trapezoidal rule to eight hours (AUC_8), the time of the second dose (chapter 6). The absorption rate constant of ifosfamide (KA) was obtained using equation 6.6.

9.3 Results

9.3.1 Ifosfamide pharmacokinetics

The pharmacokinetic data are given in the appendix (tables A6 to A23), and parameters are shown in table 9.1. The profiles from one patient are illustrated in figure 9.1. The mean absorption rate constant of ifosfamide on day 1 was 1.23 hr^{-1} (SD ± 1.01), with one patient absorbing the drug very quickly.

	Cmax (μM)	AUC_8 (μM.hr)	KA (hr^{-1})	Cmin (μM)
Ifosfamide				
Day 1				
Mean	53.1	293	1.23	-
SD	18.5	67	1.01	-
n	11	11	11	-
Day 14				
Mean	50.7	256	-	8.0
SD	25.8	118	-	6.9
n	7	7	-	7
IPM				
Day 1				
Mean	1.1	8.5	-	-
SD	0.41	2.9	-	-
n	8	10	-	-
Day 14				
Mean	1.1	8.4	-	0.17
SD	0.57	2.9	-	0.19
n	6	6	-	6

Table 9.1 a. Pharmacokinetic parameters of ifosfamide and isophosphoramide mustard.

	Cmax (μM)	AUC_8 (μM.hr)	Cmin (μM)
2-DCEI			
Day 1			
Mean	2.0	14	-
SD	0.65	5.5	-
n	10	9	-
Day 14			
Mean	4.3	27	1.5
SD	1.9	12	1.1
n	6	6	6
3-DCEI			
Day 1			
Mean	4.4	30	-
SD	2.5	14	-
n	9	11	-
Day 14			
Mean	7.5	58	3.3
SD	4.0	26	2.6
n	6	7	7

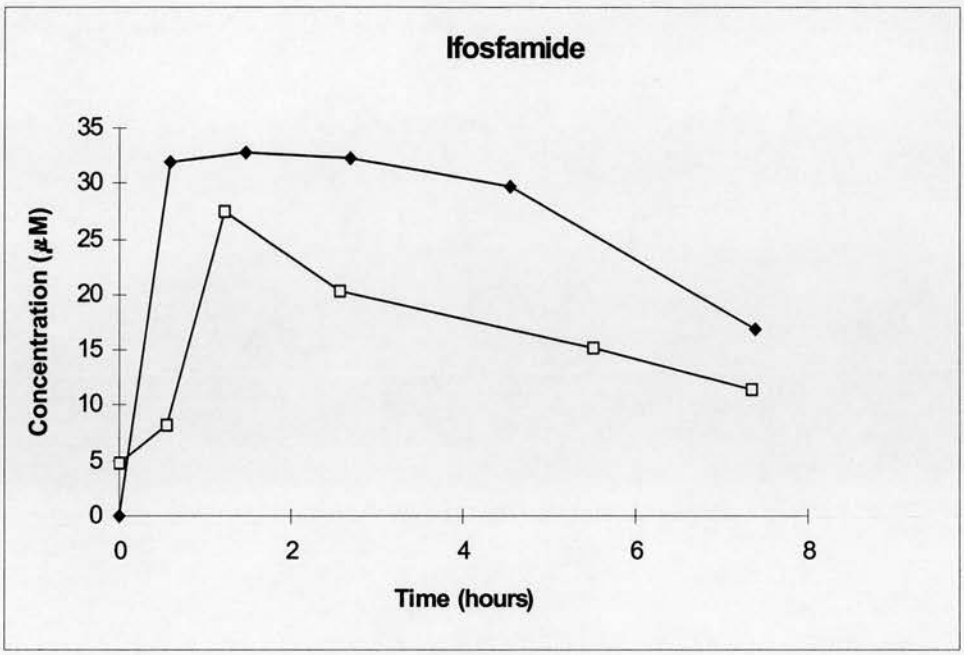
Table 9.1 b. Pharmacokinetic parameters of 2-dechloroethylifosfamide and 3-dechloroethylifosfamide.

	Cmax (μM)	AUC_8 (μM.hr)	Cmin (μM)
CIPA			
Day 1			
Mean	1.5	9.3	-
SD	0.80	3.5	-
n	11	11	-
Day 14			
Mean	2.1	15	0.45
SD	1.1	8.5	0.54
n	7	7	7
KIPA			
Day 1			
Mean	0.69	5.45	-
SD	0.44	2.44	-
n	9	10	-
Day 14			
Mean	1.04	7.74	0.17
SD	0.53	3.85	0.13
n	7	7	7

Table 9.1 c. Pharmacokinetic parameters of carboxyifosfamide and ketoifosfamide.

	Cmax (μM)	AUC_8 (μM.hr)	Cmin (μM)
CEA			
Day 1			
Mean	7.6	52	-
SD	4.0	23	-
n	9	9	-
Day 14			
Mean	7.3	53	1.3
SD	3.2	26	2.1
n	6	6	6
OXA			
Day 1			
Mean	0.68	9.7	-
SD	0.29	2.6	-
n	9	9	-
Day 14			
Mean	0.87	10.7	0.18
SD	0.42	2.9	0.30
n	6	6	6

Table 9.1 d. Pharmacokinetic parameters of chloroethylamine and 1, 3-oxazolidine-2-one.



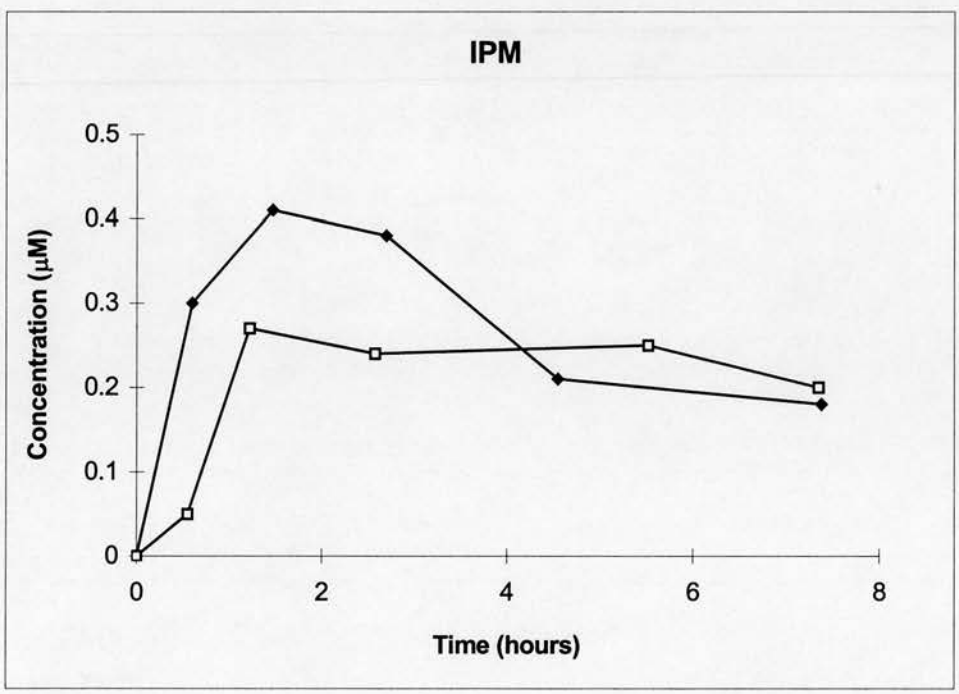
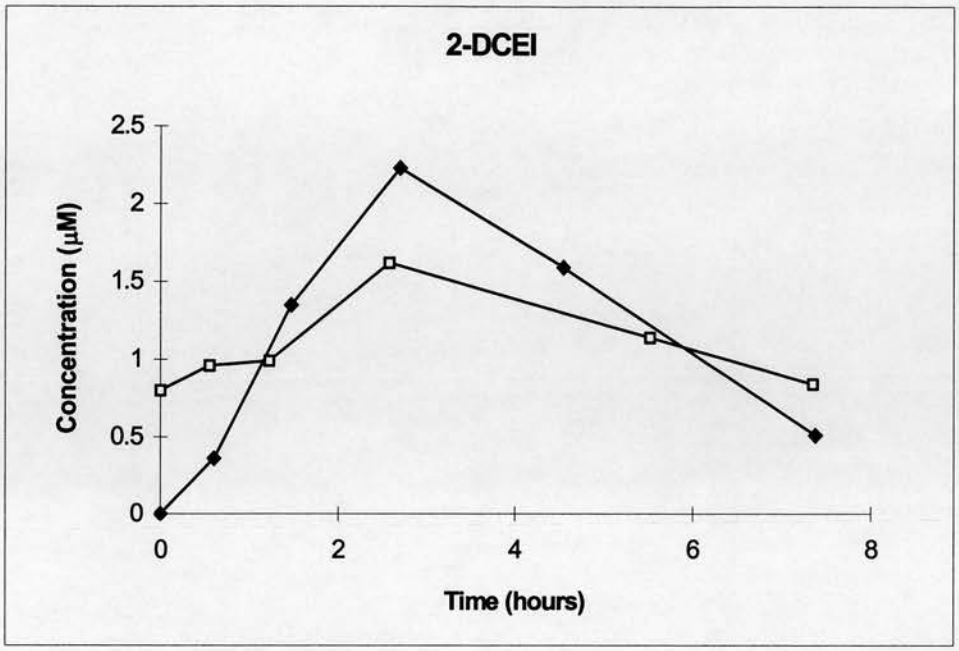


Figure 9.1 a. Plasma concentration-time profiles of ifosfamide and IPM after oral ifosfamide administration; day 1 ◆; day 14 □.



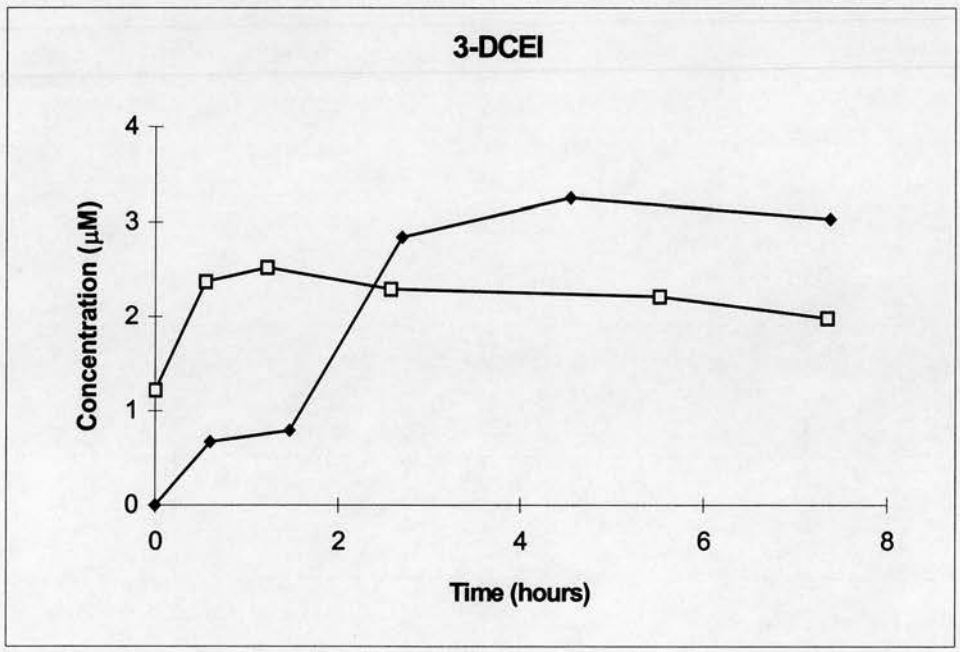
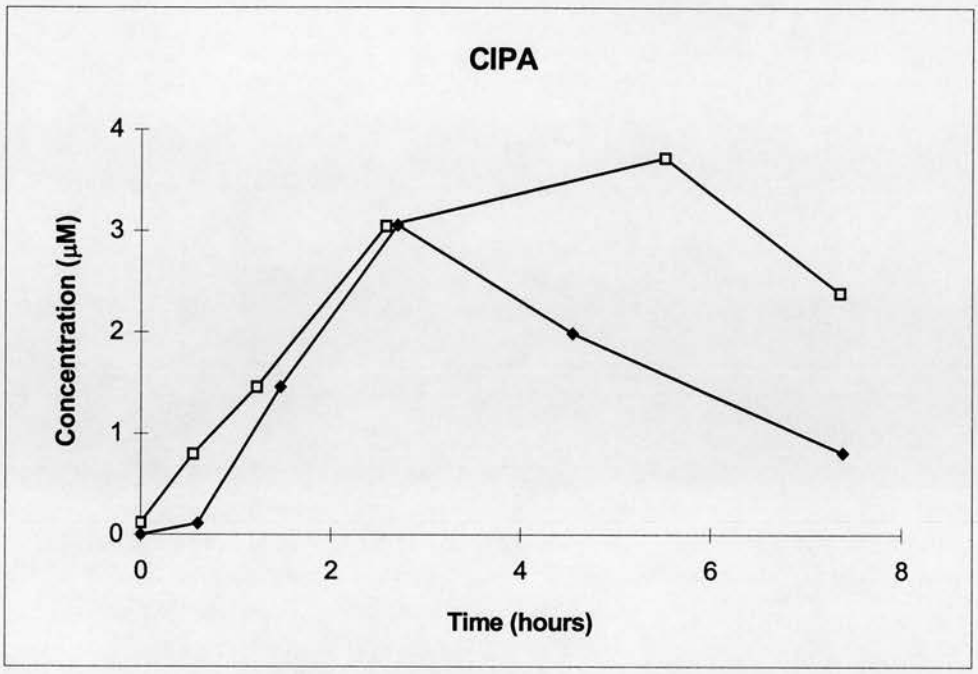


Figure 9.1 b. Plasma concentration-time profiles of 2-DCEI and 3-DCEI after oral ifosfamide administration; day 1 ◆; day 14 □.



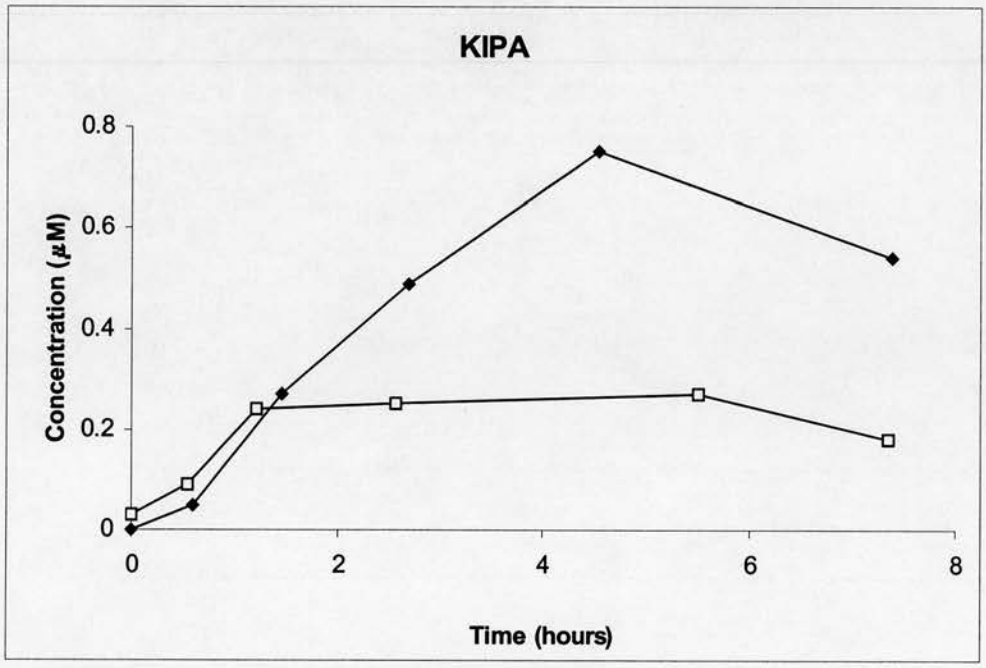
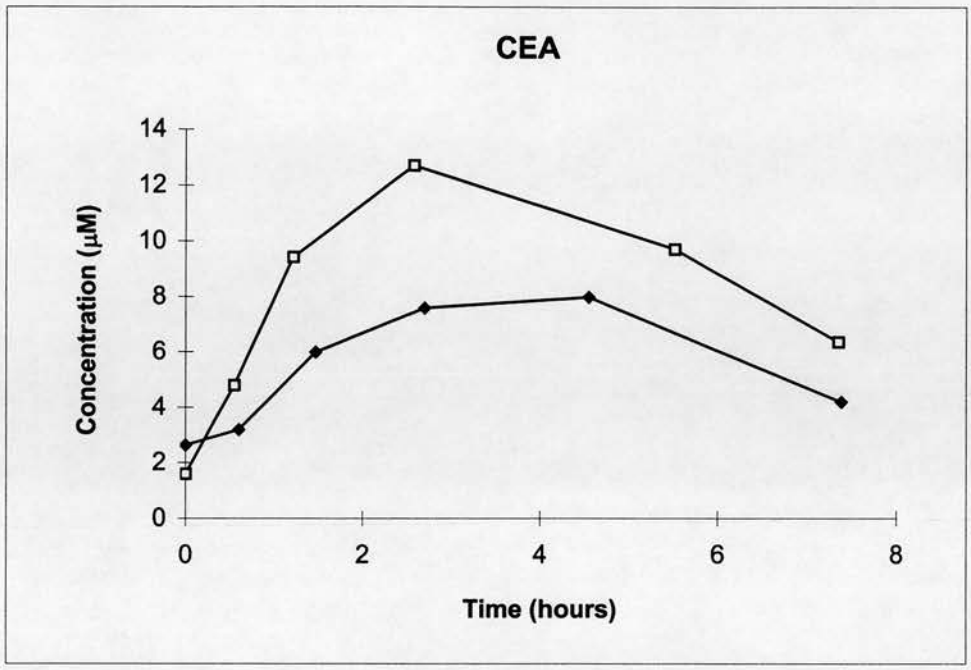


Figure 9.1 c. Plasma concentration-time profiles of CIPA and KIPA after oral ifosfamide administration; day 1 ◆; day 14 □.



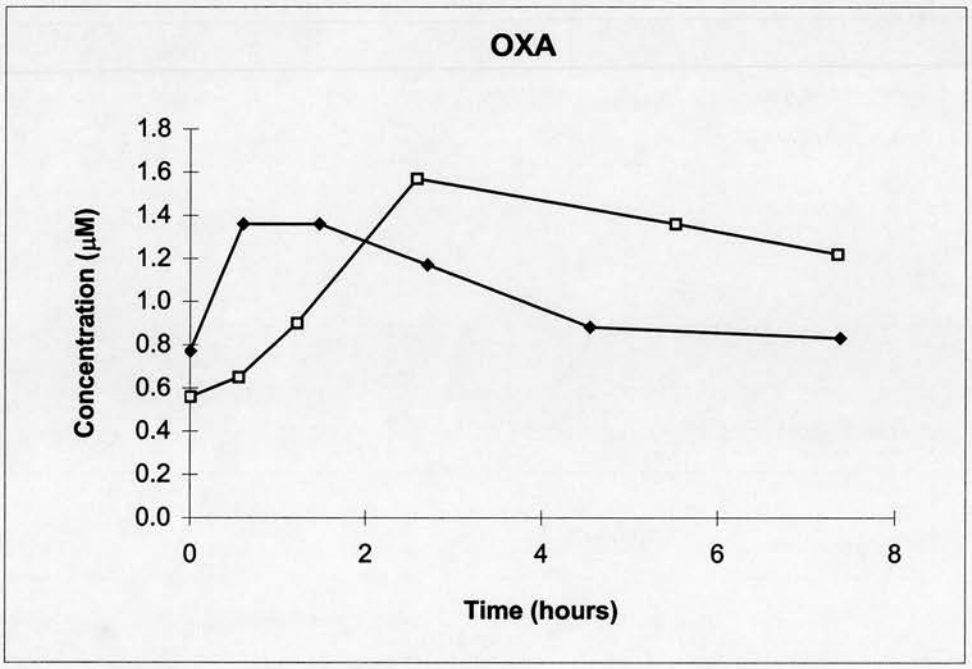


Figure 9.1 d. Plasma concentration-time profiles of CEA and OXA after oral ifosfamide administration; day 1 ◆; day 14 □.

The ratios of day 14 Cmax / day 1 Cmax are given in table A23, and the mean values illustrated in figure 9.3. The ratio for ifosfamide was less than 1, and the highest ratios were observed with KIPA and 2-DCEI. Overall, the ratios suggest increased formation and/or accumulation of metabolites, other than IPM, by day 14.

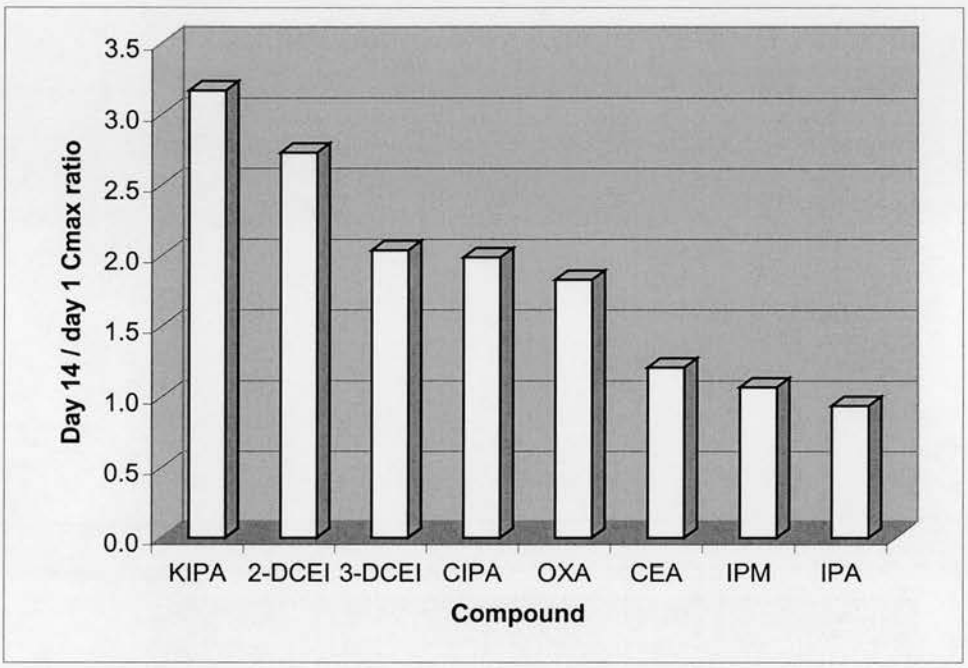


Figure 9.3. Ratio of day 14 Cmax / day 1 Cmax for ifosfamide and its metabolites (mean values).

The Cmax values on days 1 and 14 were compared, as were the AUC_8 values on days 1 and 14, using a paired t-test (table A28). The differences between day 1 and day 14 Cmax values of 2-DCEI and CIPA, and AUC_8 values of CIPA and CEA, were statistically significant.

9.3.2 Ifosfamide pharmacokinetics and neurotoxicity

The Cmax of ifosfamide and its metabolites were correlated with the alpha frequency in the EEG on days 2 and 15, using Pearsons correlation coefficient (tables A24 and A25). Percentage changes of less than 100% in EEG alpha frequency reflect increasing neurotoxicity (negative r values). A negative and positive correlation was noted between CEA Cmax and IPM Cmax respectively. The role of dechloroethylation in the development of encephalopathy is also implicated; for the dechloroethylated metabolites, the mean day 14 / day 1 Cmax ratio was greater than 2 (Figure 9.3), whilst neurotoxicity was progressive in all patients during the 14 days of treatment.

9.4 Discussion

As a limited amount of data is available from the pharmacokinetic study, only certain trends can be identified. Similar behaviour is suggested for all metabolites except IPM. There is a tendency to higher values of Cmax, implying more generation during the treatment period. During prolonged oral ifosfamide administration, metabolism is directed more to dechloroethylation, and ifosfamide undergoing activation shows a greater tendency to form CIPA and KIPA, with less quantities of IPM. However it is not possible to compare days 1 and 14 formally; there was no washout period before pharmacokinetic sampling on day 14. Furthermore, time points for sampling did not extend through the two hours preceding the next dose, hampering AUC calculations. The pharmacokinetic study was part of two larger multi-centre clinical trials of oral ifosfamide in NSCLC and cervical cancer, specifying a fourteen day period of oral ifosfamide; these clinical studies were terminated prematurely on account of the observed neurotoxicity. The pharmacokinetic study would have been improved if a three day washout period had been allowed after the first dose, and pharmacokinetic sampling had been performed on the last dose. More time points are necessary to calculate AUC_{∞}.

The scarcity of data concerning relationships between pharmacokinetic parameters and EEG impairment means that interpretation must be cautious. However some generalisations can be made. With higher ifosfamide AUC_8 values on day 1, less impairment was observed in the EEG, implying that higher rates of ifosfamide metabolism result in greater neurotoxicity. An elevated Cmax of 2-DCEI was associated with less impairment, whereas the converse was true for the Cmax of 3-DCEI. Previous studies of oral ifosfamide have not established a link between ifosfamide metabolites and neurotoxicity; Lind et al (1990b) reported a higher urinary recovery of dechloroethylifosfamide, IPM and CIPA after oral ifosfamide, compared to the intravenous route, but there was no correlation between any individual metabolite and the development of neurotoxicity.

In summary, the results suggest that increased metabolism of ifosfamide, and/or an accumulation of its metabolites, is associated with a greater impairment of EEG appearances. It is not possible to identify one particular metabolite. Statistical analysis must be guarded; the t-tests and correlations imply that the dechloroethyl metabolites and CIPA may be important, and that these require further study. The data is consistent with previous reports implicating dechloroethylation in the aetiology of neurotoxicity; the increase in Cmax of the dechloroethylated metabolites on day 14 was secondary only to that of KIPA. Neurotoxicity was progressive throughout the fourteen day period, as metabolites accumulated. Additional work is needed, with an improved study design, to allow a more complete assessment of the pharmacokinetic parameters of KIPA, 2-DCEI, 3-DCEI and CIPA during prolonged treatment with oral ifosfamide.

Chapter 10

The Formation of Chloroethylamine and 1, 3-Oxazolidine-2-one

10.1 Introduction

The metabolism of ifosfamide is described in chapter 2. However, plasma profiling studies of patients receiving ifosfamide have neglected the metabolism of IPM to CEA (figure 2.2), as predicted by the formation of NNM from CPM (Struck et al 1975). Hydrolysis of cyclophosphamide to NNM has also been described in vitro (Arnold and Klose 1960). This chapter investigates the corresponding behaviour of ifosfamide.

10.2 The hydrolysis of cyclophosphamide

In aqueous solution at 70°C, cyclophosphamide hydrolyses to NNM, N-propanolamine and phosphoric acid (Arnold and Klose 1960). Following more prolonged exposure of cyclophosphamide solutions to a temperature of 37°C, 2, 2′-dichlorodiethylamine and 2-hydroxy-tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide have been described (Arnold and Klose 1961). The latter compound is then converted to the phosphoric acid ester of 3-hydroxypropyl-amine.

10.3 The detection of chloroethylamine and 1, 3-oxazolidine-2-one

The basic metabolites CEA and OXA were extracted from plasma using ethyl acetate, after dilution with 0.05 M borate – sodium hydroxide buffer of pH 10 (chapter 5). N-chloroethyl-1, 3-oxazolidine-2-one was employed as the internal standard. Trifluoroacetylation was performed with TFAA in ethyl acetate at 70°C for two hours.

The trifluoroacetyl derivatives were dissolved in ethyl acetate and injected on to the GC column; EI+ was used for mass spectrometric analysis. In the quantitative in vitro work, extraction was performed with dichloromethane and trifluoroacetylation performed at 65°C for one hour.

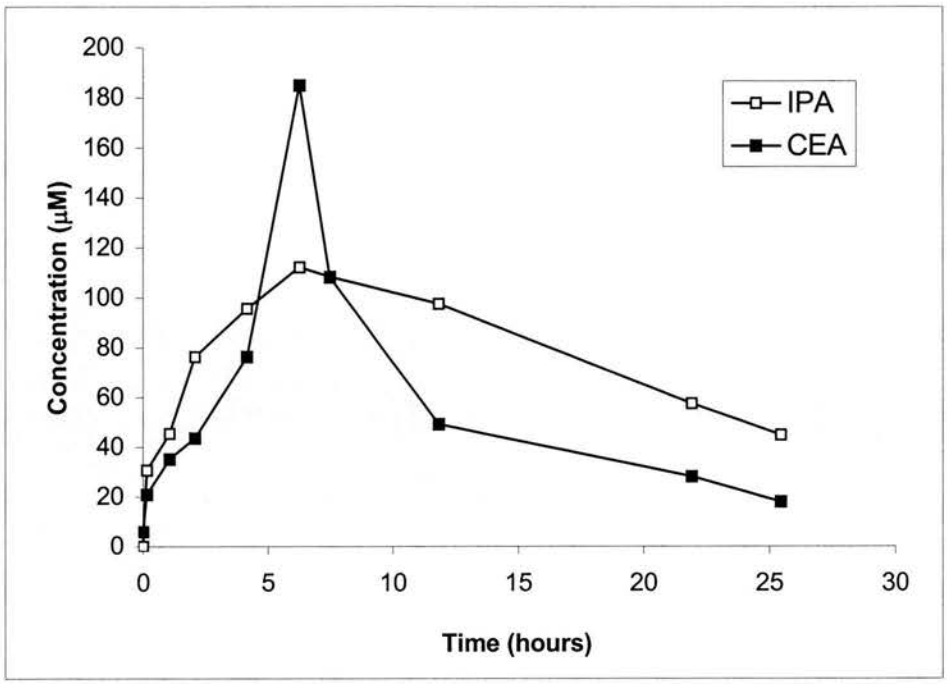
10.4 The formation of chloroethylamine and 1, 3-oxazolidone-2-one following ifosfamide administration in man

10.4.1 Patients studied

Four patients, who are also described in chapters 9 and 11, were studied. Patients AH and ML received 3 g m^{-2} ifosfamide (5.35 g and 4.7 g respectively), and 2 g m^{-2} mesna, as a six hour intravenous infusion in 500 ml 0.9% sodium chloride; intravenous boluses of 800 mg mesna were given immediately before and eight hours after the infusion. Patients CB and JW were given ifosfamide 500 mg orally as a gelatin capsule with a 300 mg film coated mesna tablet. None had received prior treatment with oxazaphosphorines. Blood samples were taken at intervals during and following the intravenous infusion for about 24 hours after completion, and for approximately six hours after oral administration. Plasma was separated immediately by centrifugation and stored at -20°C until analysis. Ifosfamide, IPM, 2-DCEI, 3-DCEI, CIPA, KIPA, CEA and OXA were determined using GC–MS as described previously (chapter 5).

10.4.2 Results

The plasma ifosfamide and metabolite profiles of patients AH and CB are shown in figure 10.1.



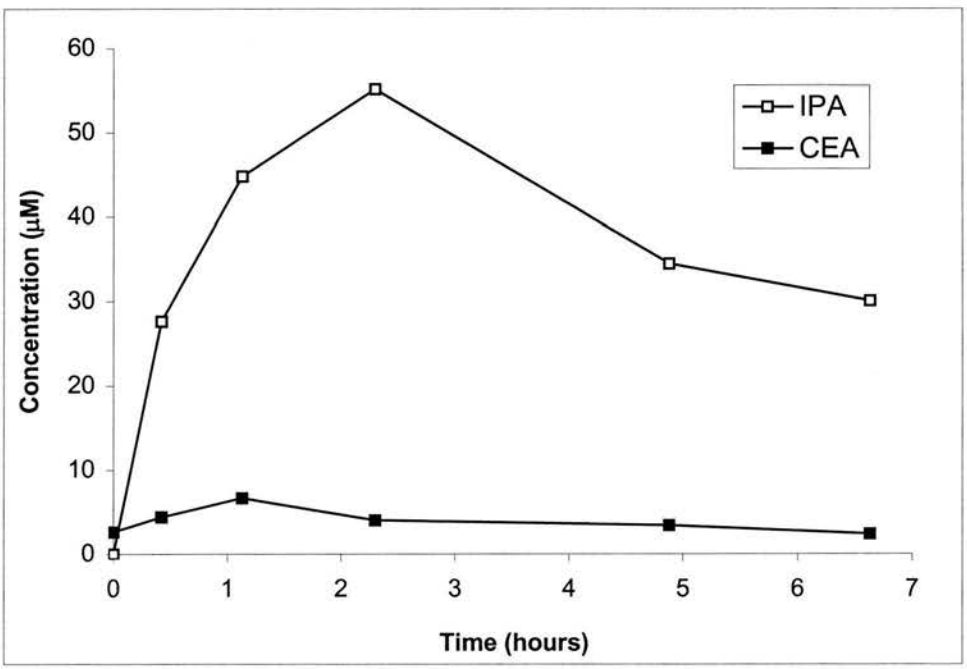
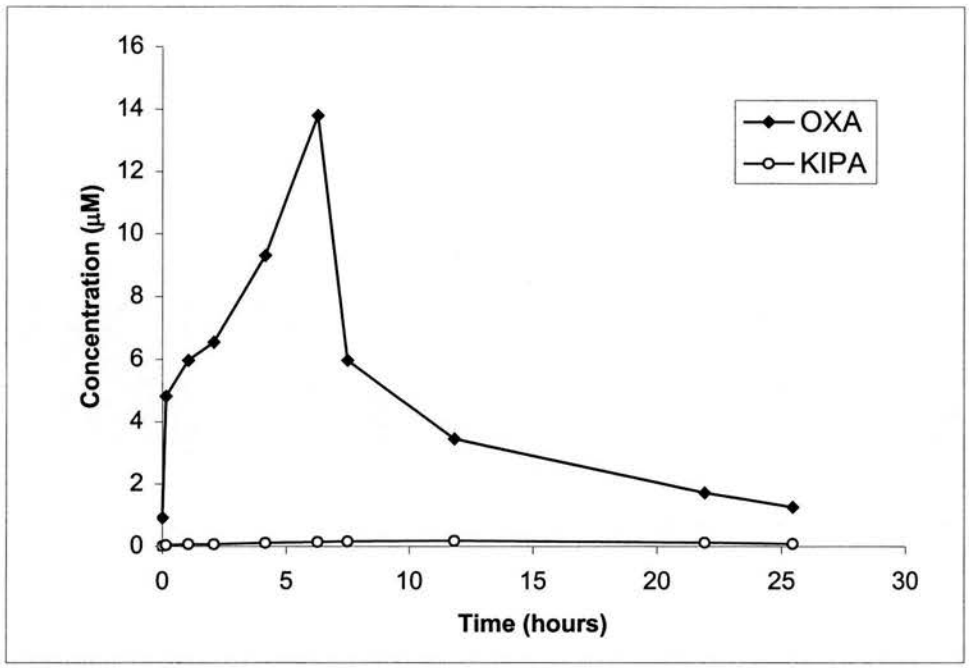


Figure 10.1 a. Plasma concentration time curves of ifosfamide and chloroethylamine following intravenous (upper graph, patient AH), and oral (lower graph, patient CB) administration.



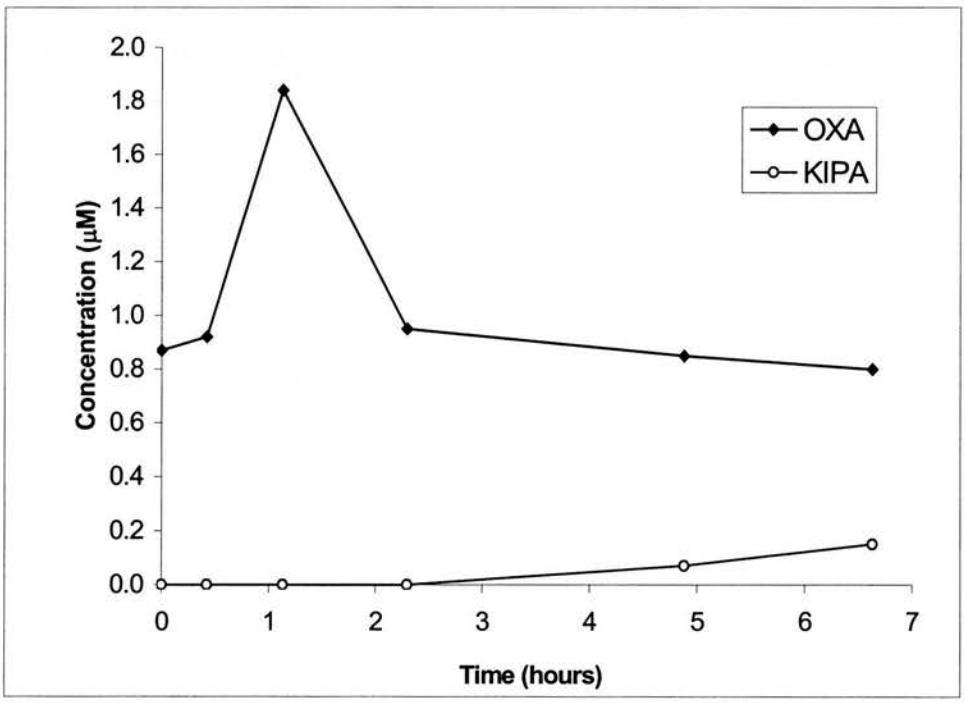
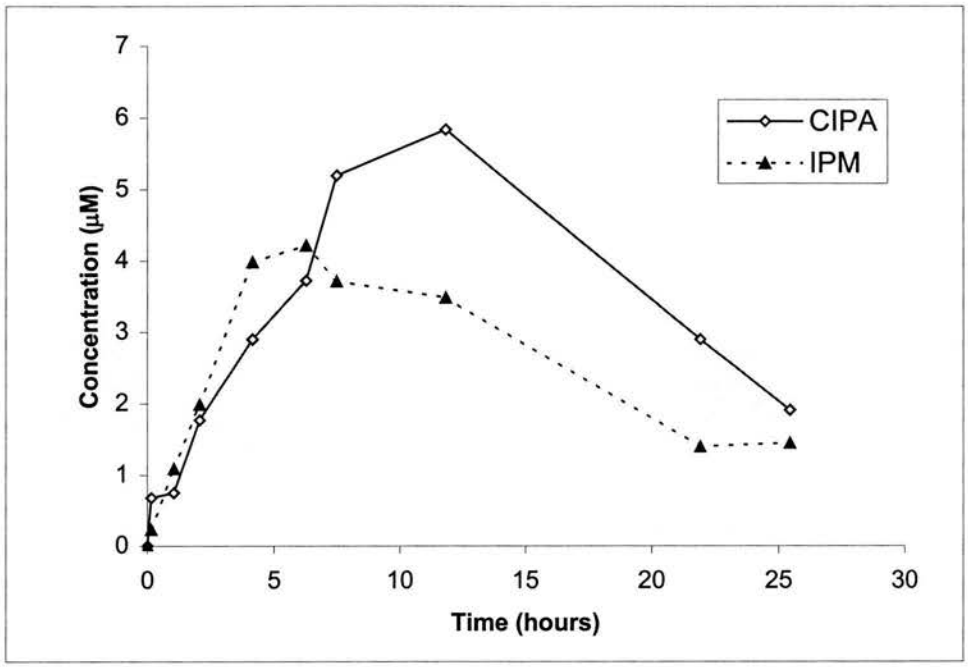


Figure 10.1 b. Plasma concentration time curves of 1, 3-oxazolidine-2-one and ketoifosfamide following intravenous (patient AH, upper graph), and oral (patient CB, lower graph) administration.



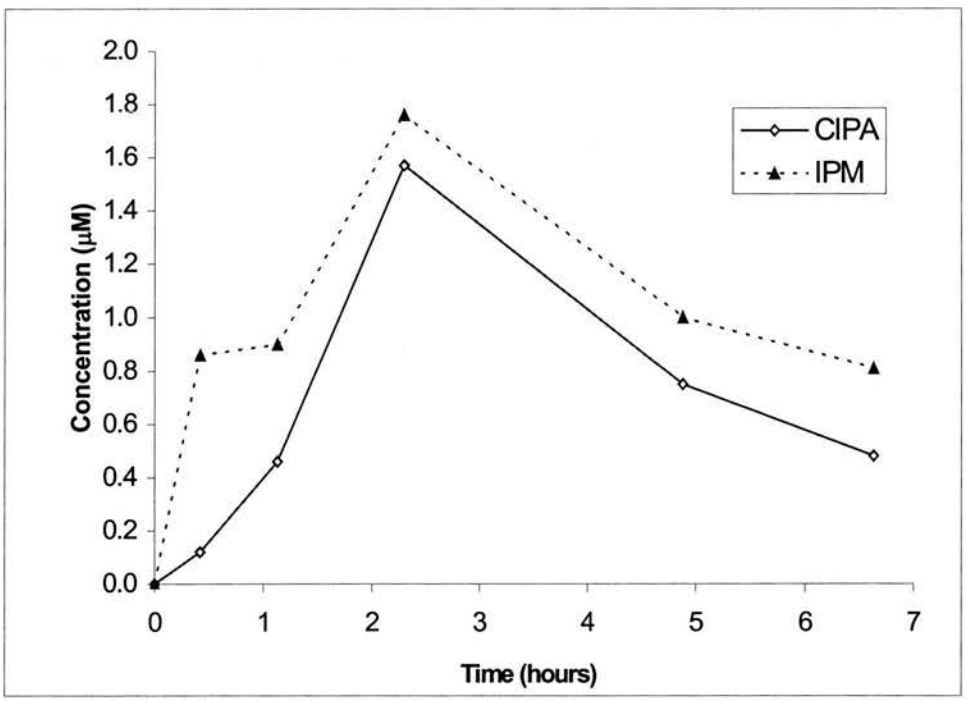
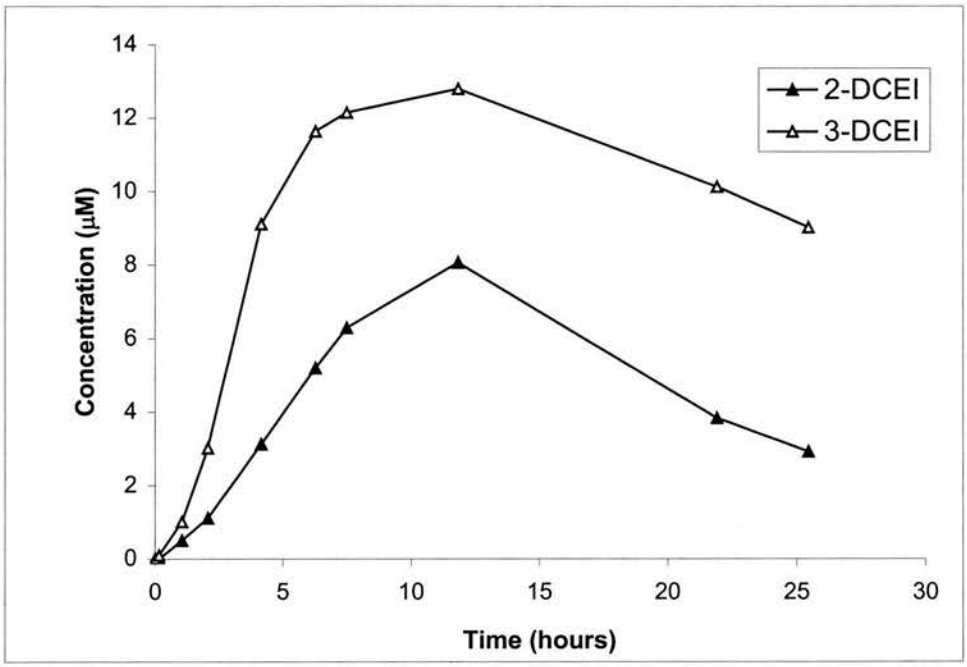


Figure 10.1 c. Plasma concentration time curves of carboxyifosfamide and isophosphoramide mustard following intravenous (patient AH, upper graph), and oral (patient CB, lower graph) administration.



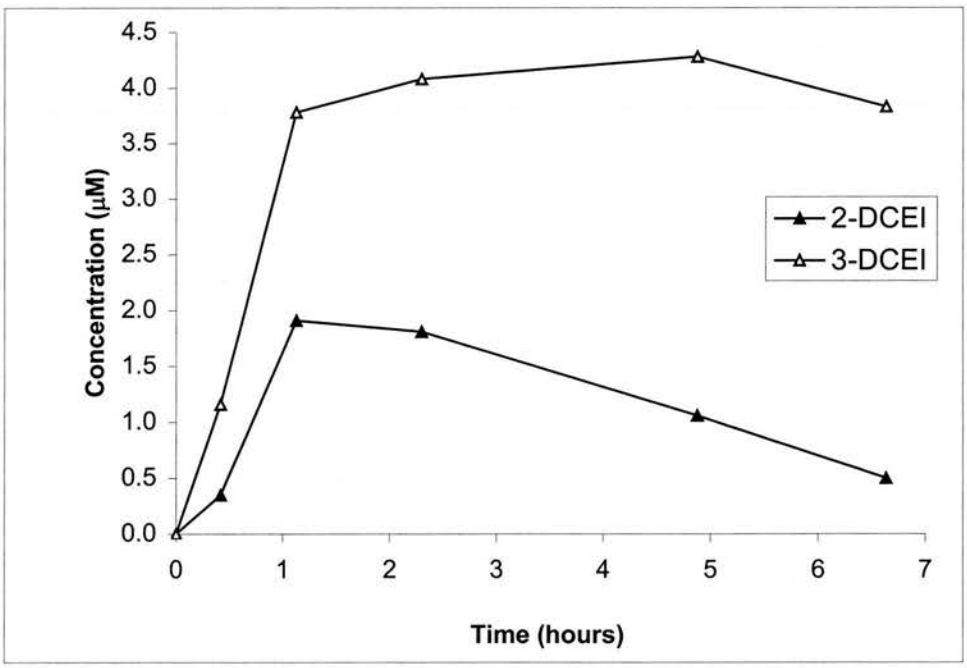


Figure 10.1 d. Plasma concentration time curves of 2-dechloroethylifosfamide and 3-dechloroethylifosfamide following intravenous (patient AH, upper graph), and oral (patient CB, lower graph) administration.

Peak levels of 2-DCEI, 3-DCEI and CIPA occurred about six hours after the end of the intravenous infusion, and only a small amount of KIPA was seen. Peak concentrations of IPM, CEA and OXA coincided with the end of the ifosfamide infusion. With oral administration, the highest concentrations of ifosfamide, IPM and CIPA were observed after approximately two hours; concentrations of CEA and OXA peaked before that of ifosfamide.

Unexplained small quantities of CEA (0.21 $\mu g\ ml^{-1}$ [2.6 μM] to 0.47 $\mu g\ ml^{-1}$ [5.9 μM]) and OXA (0.076 $\mu g\ ml^{-1}$ [0.87 μM] to 0.08 $\mu g\ ml^{-1}$ [0.92 μM]) were present in the plasma before ifosfamide administration, and similar amounts were seen in plasma taken from the blood bank; neither compound was detected in 0.9% sodium chloride solutions stored in the same plastic tubes and conditions as the patient plasma samples. It is interesting that aliphatic amines such as trimethylamine are naturally present in human biofluids (Ayesh et al 1993).

Following intravenous dosing, much higher plasma concentrations of CEA were found compared to IPM, suggesting the presence of an additional route of CEA formation. A similar difference was observed between patients ML and JW; after intravenous infusion the peak concentration of CEA was 9.73 $\mu g\ ml^{-1}$ (122 μM) and of ifosfamide 20.37 $\mu g\ ml^{-1}$ (78 μM), compared to respective values of 0.43 $\mu g\ ml^{-1}$ (5.4 μM) and 10.45 $\mu g\ ml^{-1}$ (40 μM) following oral administration. The plasma molar AUC_{∞} values of ifosfamide and its metabolites for patient AH are given in table 10.1.

10.5 The formation of chloroethylamine and 1, 3-oxazolidine-2-one following the hydrolysis of ifosfamide in vitro

Following the observation of relatively high levels of CEA compared to IPM after intravenous ifosfamide, in vitro work was performed to explore this further. Aqueous solutions of ifosfamide, 1000 $\mu g\ ml^{-1}$ (3.8 mM) and 100 $\mu g\ ml^{-1}$ (0.38 mM) were buffered at pH 4, 7 and 10, maintained at 40°C for five hours, and an aliquot extracted with 6 ml of dichloromethane. The solvent was evaporated, and the dry

	IPA	2-DCEI	3-DCEI	IPM	CIPA	KIPA	CEA	OXA
AUC_{∞}	2665	160	529	86	110	15	1570	128
% of IPA		6	20	3	4	1	59	5

Table 10.1. AUC_{∞} (μM hr) of ifosfamide and its metabolites, also expressed as a percentage of the ifosfamide AUC_{∞}.

residue treated with diazomethane in ether, the solvent again evaporated, and the residue dissolved in 500 μl of methanol. The methanol solution was then injected into the GC–MS. In all six samples three peaks were detected: using EI+ ionisation, the first was identified as phosphoric acid methyl ester; the second as 3-(2-chloroethyl)-2-methoxy-tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide; and the third as ifosfamide. CEA was also detected in all six solutions, as described above. These findings suggest that hydrolysis of ifosfamide to CEA and 3-(2-chloroethyl)-2-hydroxy-tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide (II) is taking place. In a further experiment, an aqueous solution of ifosfamide (2000 μg ml^{-1}; 7.6 mM) was maintained at 40°C for 12 hours. An aliquot was again extracted with dichloromethane, dried over sodium sulphate, filtered, and evaporated to a residual volume of 300 μl. An equal volume of trifluoroacetic anhydride was then added, and the mixture heated at 65°C for one hour. After evaporation to dryness, the residue was dissolved in 100 μl of ethyl acetate and the resulting solution injected into the GC–MS. Using EI+ ionisation the trifluoroacetyl derivatives of ifosfamide, CEA and 2-chloroethyl-3-hydroxypropyl-amine were observed. The hydrolysis of ifosfamide is shown in figure 10.2.

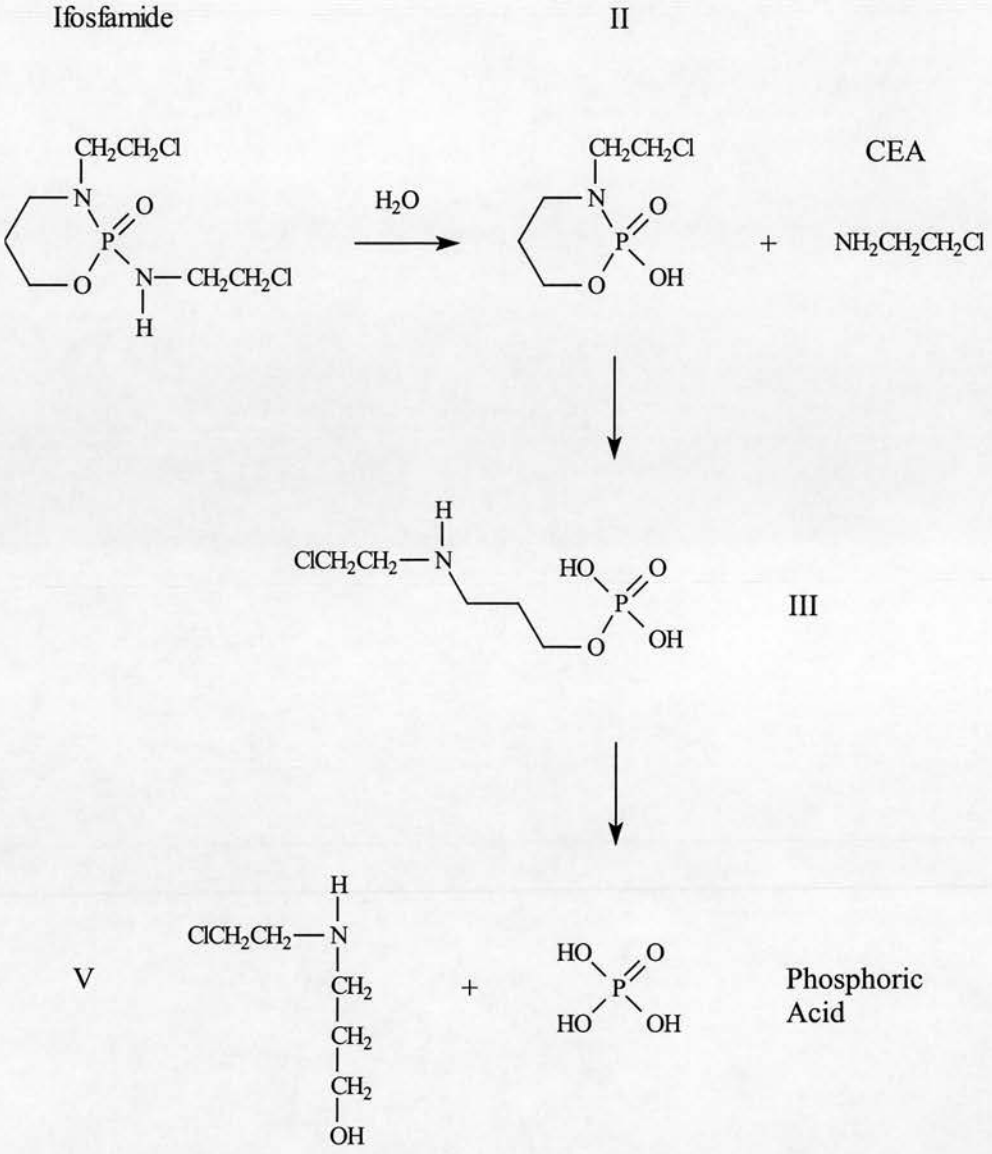


Figure 10.2. Hydrolysis of ifosfamide; II = 3-(2-chloroethyl)-2-hydroxy-tetrahydro-2H-1, 3, 2-oxazaphosphorine-2-oxide; III = Phosphoric acid ester of 2-chloroethyl-3-hydroxypropyl-amine; V = 2-chloroethyl-3-hydroxypropyl-amine.

In view of these findings, quantitative analyses of CEA and ifosfamide were performed in the following systems: two aqueous solutions of ifosfamide containing 53 μg ml^{-1} (203 μM) (A) and 5.2 μg ml^{-1} (20 μM) (B) maintained at 37°C for six hours; whole

blood spiked with 1 μg ml^{-1} (3.8 μM) of ifosfamide and incubated at 37°C for 2.5 hours; and the infusion given to patient AH, prepared once more under the same conditions, and allowed to drain from the bag at the same rate and temperature (23°C) as during administration. Aliquots of aqueous solution, infusate and plasma were sampled at regular intervals, and analysed following trifluoroacetylation in dichloromethane, as described above. In all three systems, appreciable amounts of CEA were present at zero time, as expressed by CEA/ifosfamide ratios (% w/w; % molar) of 21.3; 69.9 (A), 17.3; 56.8 (B), 16.8; 55.2 (plasma), and 13.6; 44.7 (infusate). Significant chemical hydrolysis of ifosfamide occurs at higher concentrations in aqueous solution (figure 10.3), the ratio reaching 45.6% (150% molar) in solution A after six hours compared with 26.0% (85.4% molar) in solution B. The change over time in the cooler infusate was minimal, with a six hour w/w ratio of 16.2% (53.2% molar), and one of 18.9% (62.1% molar) in whole blood after 2.5 hours suggests a lack of enzymatic hydrolysis.

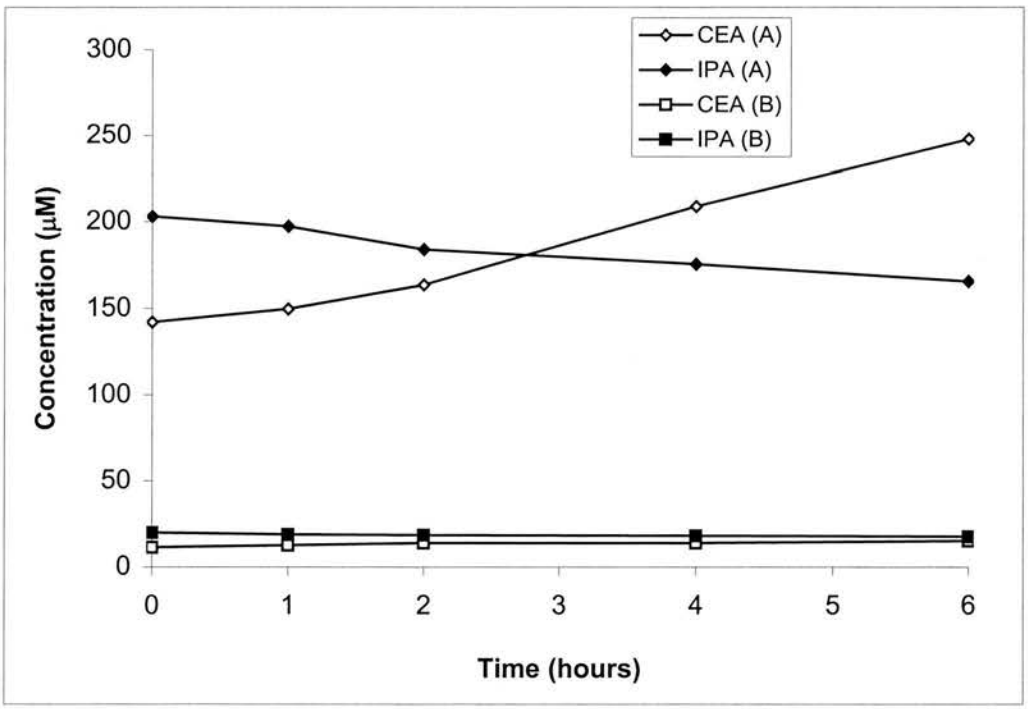


Figure 10.3. In vitro hydrolysis of ifosfamide in aqueous solution.

10.6 Discussion

Significant quantities of NNM have been reported following the intravenous administration of cyclophosphamide, and as an explanation the metabolism of carboxyphosphamide has been postulated (Jardine et al 1978). However, the plasma profiles obtained after ifosfamide administration do not indicate CIPA as a source of CEA; peak w/w CEA/ifosfamide ratios of 50.2% (165% molar) and 50.1% (164% molar) were obtained after intravenous administration, compared to corresponding w/w values of 4.9% (16.1% molar) and 8.2% (26.9% molar) after oral dosing. The relatively high levels of CEA generated after intravenous infusion, with a curve following the ifosfamide plasma profile, infer that significant hydrolysis of ifosfamide itself is occurring, either before or after direct injection into the circulation. This probably reflects the nature of the hydrolysis reaction, the rate of which is dependent on the concentration of ifosfamide and therefore the administered dose.

In terms of plasma AUC_{∞}, an ifosfamide value of 2665 μM hr obtained in this study, after a six hour infusion of 3 g m^{-2} ifosfamide, is consistent with published results. Using ifosfamide infusions of 1.5 g m^{-2} over 20 to 60 minutes, ifosfamide AUCs of 1520 to 2344 μM hr have been reported (Kurowski et al 1991; Kurowski and Wagner 1993; Lewis et al 1990; Lind et al 1989a), whilst figures of 2240 μM hr and 2309 μM hr have been observed after 20 minute infusions of 2 g m^{-2} and 3 g m^{-2} ifosfamide respectively (Wagner and Drings 1986; Aeschlimann 1998).

The plasma AUC_{∞} of CEA represents 59% of the ifosfamide dose. The amount generated by the breakdown of IPM is negligible, suggesting that the majority of CEA has an alternative origin. Dechloroethylation to 3-DCEI accounts for 20% of the ifosfamide dose, and it is conceivable that 3-DCEI could be further metabolised to CEA. Hydrolysis of 3-DCEI in vitro can liberate CEA, but with only 2% degradation at pH 6.8 after 28 days (Gilard et al 1999). However compounds E, D and A (Figure 10.4) represented approximately 10% of 2-DCEI excreted over 24 hours in the urine of patients receiving 3 g m^{-2} of ifosfamide as a three hour infusion (Gilard et al 1993); 2, 3-didechloroethylifosfamide, although not detected, was

Figure 10.4. Breakdown products of the dechloroethylated metabolites of ifosfamide.

thought to be the precursor of compounds E and D, which would be formed by hydrolysis. Unknown metabolites represented 18% of the excreted dose in this study. In patients treated with cyclophosphamide 60 mg kg^{-1} day^{-1} as a three hour infusion, compounds C, D and F represented approximately 22% and 57% of 3-DCEI excreted in urine at pH 5.8 and 5.1 to 5.3 respectively (Joqueviel et al 1998). Carboxycyclophosphamide was metabolized into two phosphoric acid esters, with the generation of NNM. Urinary excretion of these esters represented approximately 10% of the carboxycyclophosphamide excreted, but was pH dependent, reflecting the known instability of this metabolite. Nevertheless, such a metabolic route acting on CIPA would provide another potential source of CEA. Aeschlimann et al measured the urinary excretion of CEA using GLC, following three single daily intravenous or oral doses of ifosfamide (Aeschlimann et al 1998). The urinary recovery of CEA was expressed as a percentage of the total ifosfamide dose, assuming that one mole of ifosfamide generates two moles of CEA. The methodology was unable to detect CEA in plasma. There was no difference in the recovery of CEA in urine following intravenous or oral administration (4.1% to 12.7% compared to 3.2% to 6.5% respectively). However the interpatient variability of urinary excretion of CEA was large. The authors postulated that CEA could be metabolized to chloroacetaldehyde, as it is a substrate of plasma amine oxidase.

The in vitro work suggests that significant chemical hydrolysis of ifosfamide can occur in aqueous solution (figure 10.3). However, other studies have reported that ifosfamide is stable in aqueous solution at ambient temperature for nine days (Radford et al 1991), and at concentrations of 0.6 mg ml^{-1} and 20 mg ml^{-1} in 0.9% sodium chloride for one week at 30°C (Trissel 1992). Kaiser et al found that in the most stable pH range of 5 to 9, the half-life of ifosfamide was 20 hours at 70°C (Kaiser et al 1991a).

Further work by Gilard et al (1997) has not confirmed the results of the in vitro work described in this chapter, and has suggested that the source of the high CEA levels is degradation of ifosfamide during the derivatisation procedure. Using NMR analysis, they studied the in vitro derivatisation of ifosfamide with TFAA under three reaction conditions: ethyl acetate in a stoppered tube; dichloromethane in a stoppered tube;

and dichloromethane in a round bottomed flask fitted with a cooling system. There was no degradation of a 3.8 mM ifosfamide solution, at pH 4, 7 or 10, maintained at 40°C for five hours. Similarly, at pH 7, degradation of a more concentrated solution of ifosfamide (38 mM) was not seen after 12 hours at 40°C, or 6 hours at 37°C. At pH 4, after six hours at 37°C, a 38 mM ifosfamide solution suffered a 1.1 molar % degradation, which reached 3.9 molar % after 24 hours. The major degradation product was compound III (figure 10.3).

When derivatisation was performed in ethyl acetate, the trifluoroacetyl derivative of ifosfamide was the predominant constituent in the 19F NMR spectrum (representing more than 95 molar % of initial ifosfamide), and the total recovery was quantitative. TFA-CEA was not observed. TFAA derivatisation in dichloromethane, without controlling solvent evaporation, led to a maximum total recovery of 35 molar % of the initial ifosfamide, probably as a result of evaporation of the TFA derivatives. TFA-IF represented less than 1 molar % of the initial ifosfamide, whereas TFA-CEA was observed in approximately ten fold higher amounts. When the TFA reaction in dichloromethane was performed in cool conditions, thereby preventing solvent evaporation, the overall recovery was nearly quantitative (80 molar %). The authors concluded that ifosfamide degradation is dependent on the method of derivatisation; the compound is not easily derivatised by TFAA in dichloromethane, and the combination of heating, solvent evaporation, and the presence of trifluoroacetic acid in the medium can degrade ifosfamide with the formation of CEA.

The presence of appreciable quantities of CEA at zero time in the four systems studied (solutions A and B, plasma and infusate) indicate decomposition during analysis. However in the more concentrated solution A, the CEA/ifosfamide molar ratio changed from 69.9% at baseline to 150% after six hours at 37 °C (figure 10.3), implying additional decomposition, unrelated to analytical technique, during this time period.

CEA is an important product of ifosfamide degradation and appears to be formed predominantly by hydrolysis. Ethyl acetate was used as a solvent for derivatising

ifosfamide and its metabolites in the analysis of plasma from the in vivo study, whereas in the in vitro work dichloromethane was employed. Subsequent work by Gilard et al has suggested that the use of dichloromethane leads to degradation of ifosfamide with erroneously high levels of CEA (Gilard et al 1997). Although the high CEA levels found in vitro may be spurious, degradation of ifosfamide during analysis of plasma cannot fully explain the differences in CEA levels following intravenous and oral administration; the same analytical procedure was used, employing ethyl acetate as a solvent for the derivatisation with TFAA. Furthermore, an ifosfamide AUC of 4235 μM hr, assuming all the CEA generated is produced by ifosfamide breakdown during analysis, is perhaps higher than anticipated for the dose and infusion time of the ifosfamide administered, and the in vivo CEA curve (figure 10.1a) is not exactly parallel to the ifosfamide curve, suggesting mechanisms of CEA formation in addition to analytical artifact.

The high levels of CEA in vivo may remain an accurate reflection of changes occurring after intravenous ifosfamide administration. It is clear, however, that further work on the generation of CEA in vivo is required.

Chapter 11

The Uptake of Ifosfamide and its Metabolites by Erythrocytes

11.1 Introduction

The main site of formation of IPM, and its predominant transport form within the circulation, is not certain. Two possibilities have been envisaged; firstly, the transport of 4-hydroxyifosfamide to tumour cells, which then convert this compound to IPM, or secondly the formation of IPM, primarily within the liver, but also within other tissues capable of activating ifosfamide, followed by transport within the circulation and direct penetration of tumour cells. The relative contribution of each of these mechanisms will influence the success of any attempt to administer IPM itself as a chemotherapeutic agent, a potentially advantageous manoeuvre, which should not lead to haemorrhagic cystitis or neurotoxicity.

Consequently, an improved knowledge of the processes of distribution of ifosfamide and its metabolites is needed. Blood consists of plasma and cells, and the vast majority of the cells are erythrocytes (approximately 45% of total blood volume). It is becoming clear that the erythrocyte can be a significant carrier of substances in the circulation, but there is no data concerning the distribution of ifosfamide and its metabolites throughout whole blood in humans.

New developments in the bioanalysis of the oxazaphosphorines have provided methods for the separation and accurate measurement of many metabolites in plasma (chapter 5), and improved techniques of erythrocyte isolation have facilitated the study of the red cell compartment (chapter 7). The work described in this chapter investigates the distribution of ifosfamide and seven of its metabolites in the plasma and red cells of patients receiving an infusion of ifosfamide.

11.2 Methods

11.2.1 Patients

Five patients were studied during their first course of chemotherapy, and three additionally through their second course of treatment. 3 g m^{-2} ifosfamide was given as a six hour infusion, with mesna as a uroprotector. Three patients also received mitomycin C and cisplatin, and one patient was also given adriamycin.

11.2.2 Separation of red cells

Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 7, 9, 12, 24 and 30 hours after commencing the ifosfamide infusion. The erythrocyte and plasma fractions were separated immediately using the MESED instrument (chapter 7), and stored at -20°C. This technique allows the isolation of a known volume of almost pure red cell sediment, without disturbing the equilibrium normally present between the erythrocyte and plasma. The harvested erythrocytes were haemolysed totally with de-ionised water.

11.2.3 Determination of ifosfamide and its metabolites

The concentrations of ifosfamide, IPM, 2-DCEI, 3-DCEI, CIPA, KIPA, CEA and OXA in the plasma and erythrocyte fractions were measured using GC–MS (chapter 5).

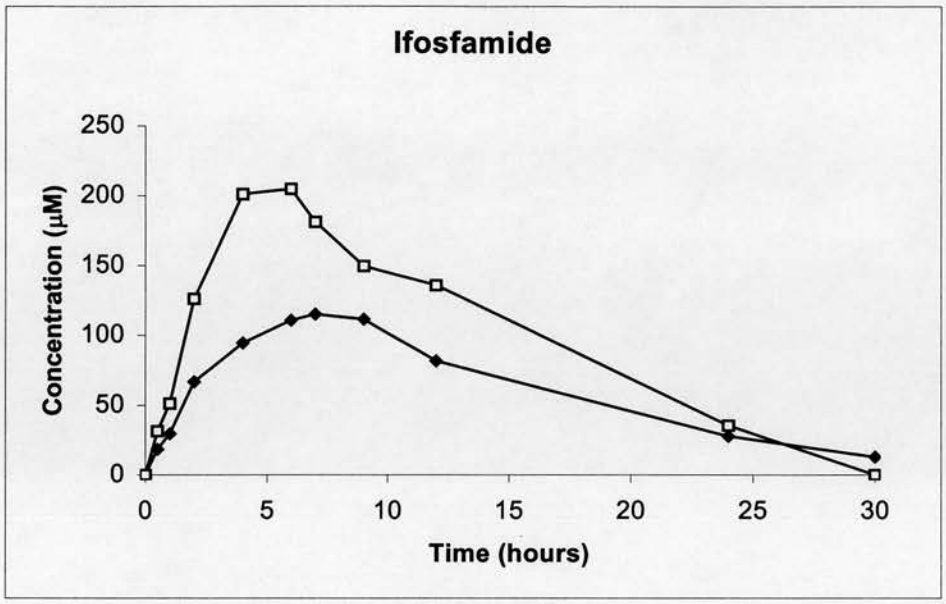
11.3 Results

The pharmacokinetic data are given in the appendix (tables A26 a-h and A27 a-b). The mean concentration-time curves of ifosfamide and its metabolites, in plasma and erythrocytes, are shown in figure 11.1 a-d. All compounds entered the red cell

compartment. Erythrocyte/plasma ratios at six hours, corresponding to the end of the ifosfamide infusion, are given in table 11.1, and the mean values were greater than one for all compounds. Comparing erythrocytes and plasma, using a paired t-test, there were significantly higher concentrations of IPM ($p = 0.007$), CIPA ($p = 0.004$) and ifosfamide ($p = 0.004$) in erythrocytes. Such differences were not detected for 2-DCEI, 3-DCEI, KIPA, CEA or OXA. Considering all concentrations after six hours, significant differences between erythrocytes and plasma were maintained for IPM ($p = 0.0005$) and CIPA ($p = 0.0007$), but not for ifosfamide itself ($p = 0.0773$).

In terms of the AUC, as calculated by the trapezoidal rule, the mean ratio of erythrocyte (AUC_E) to plasma (AUC_P) was also greater than unity in all cases (table 11.2). The difference between AUC_E and AUC_P was most evident for CIPA ($p = 0.0002$), ifosfamide ($p = 0.0003$) and IPM ($p = 0.003$), but not significant for the other metabolites (table 11.3).

The mean plasma ifosfamide half-life was 7.1 hours (SD ± 2.5), mean plasma clearance 12.96 L hr^{-1} (SD ± 3.81), and mean plasma Vss 127 L (SD ± 26).



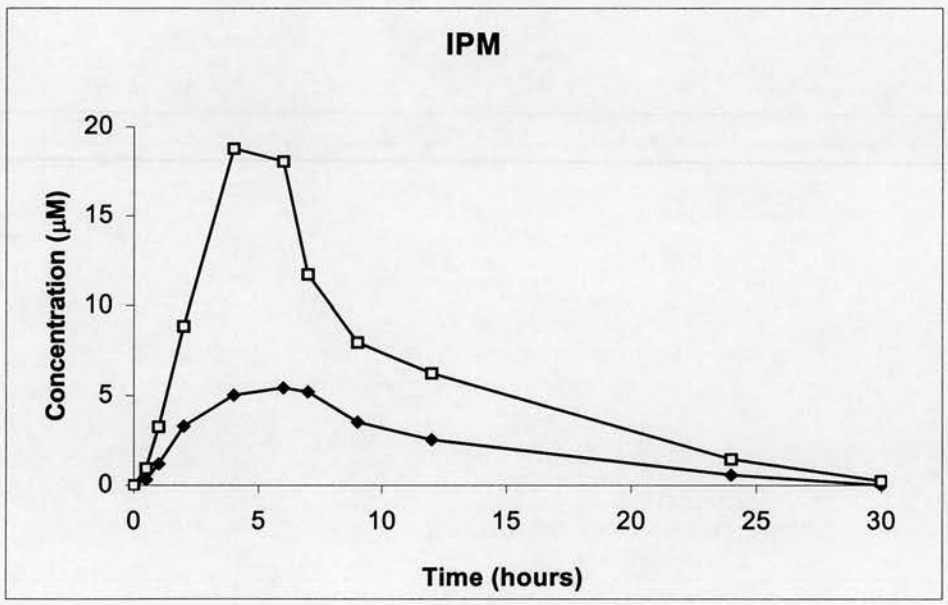
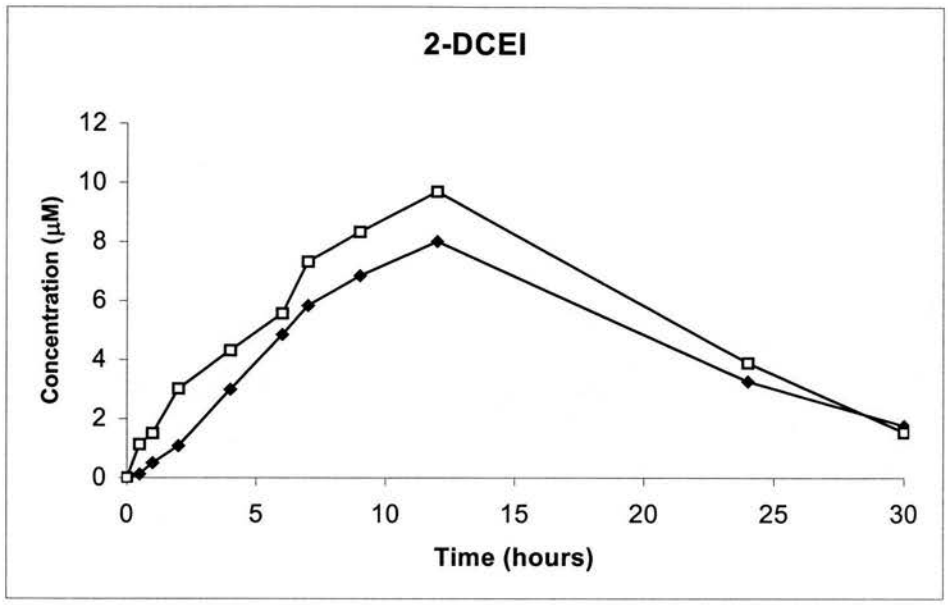


Figure 11.1 a. Mean concentration-time profiles for ifosfamide and isophosphoramide mustard; □ erythrocytes; ◆ plasma.



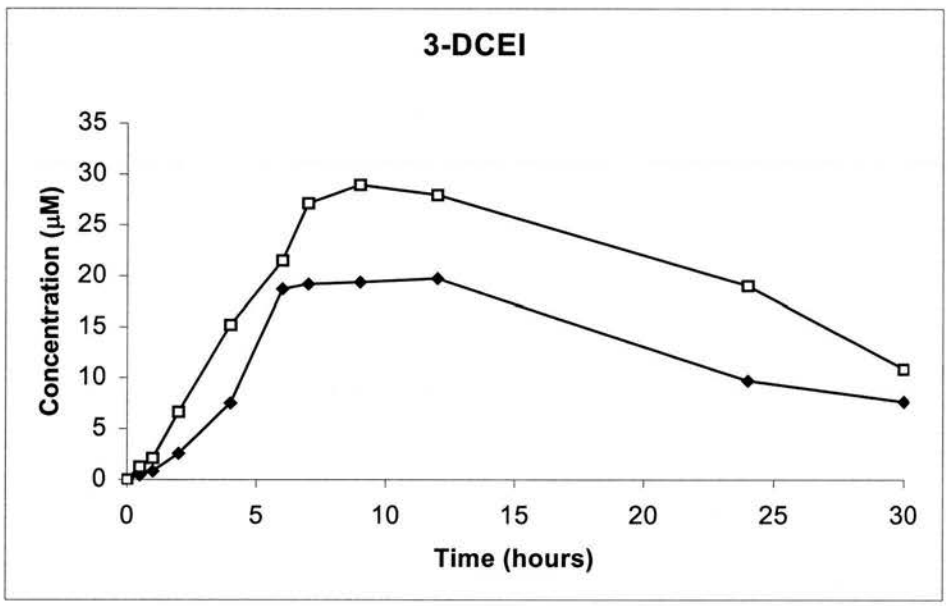


Figure 11.1 b. Mean concentration-time profiles for 2-dechloroethylifosfamide and 3-dechloroethylifosfamide; □ erythrocytes; ◆ plasma.

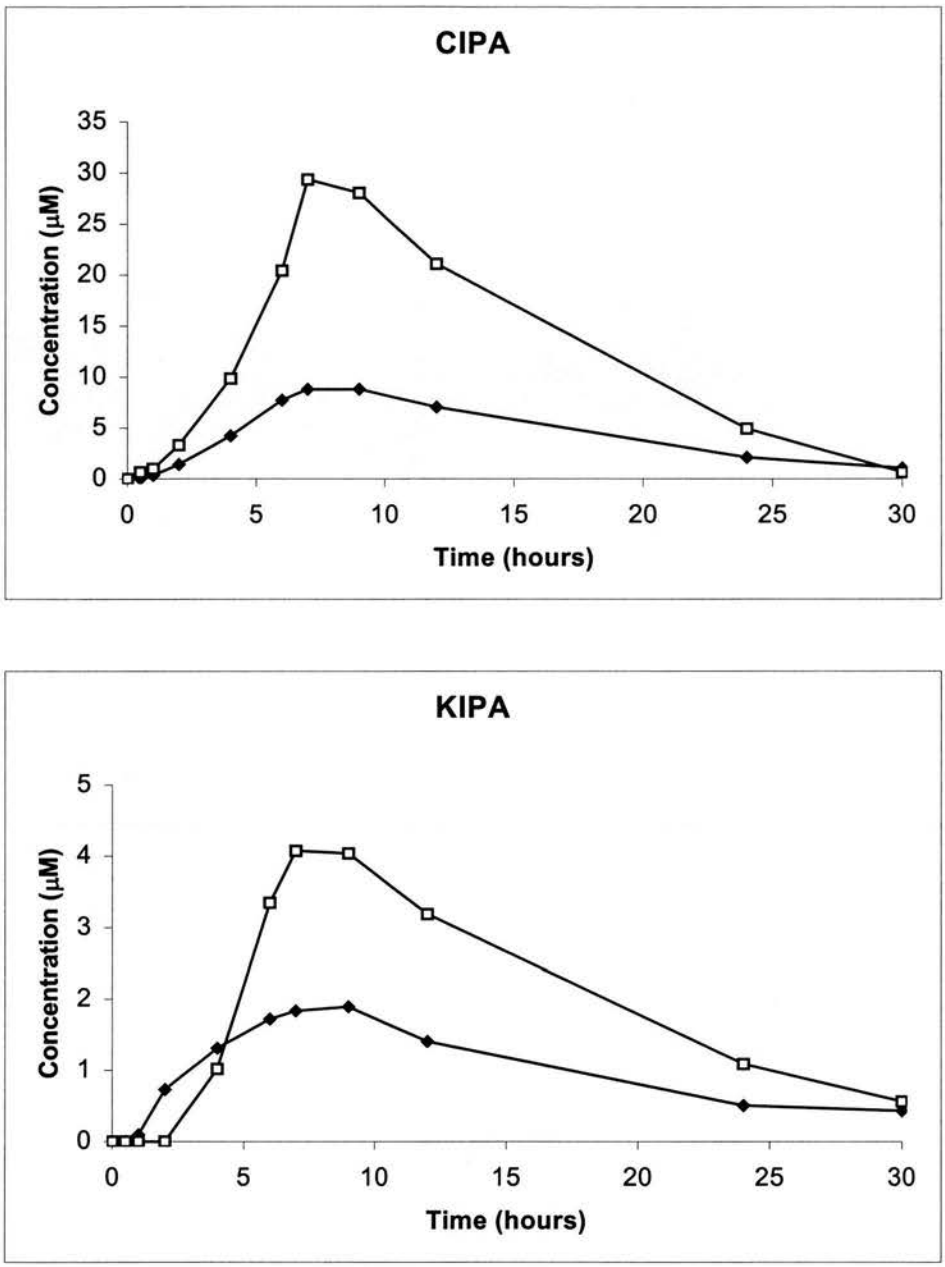
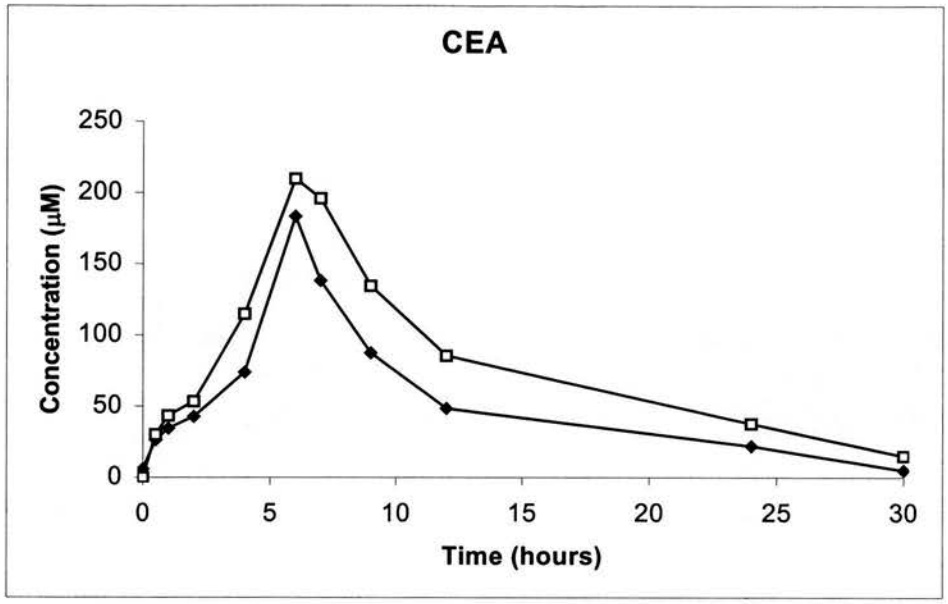


Figure 11.1 c. Mean concentration-time profiles for carboxyifosfamide and ketoifosfamide; □ erythrocytes; ◆ plasma.



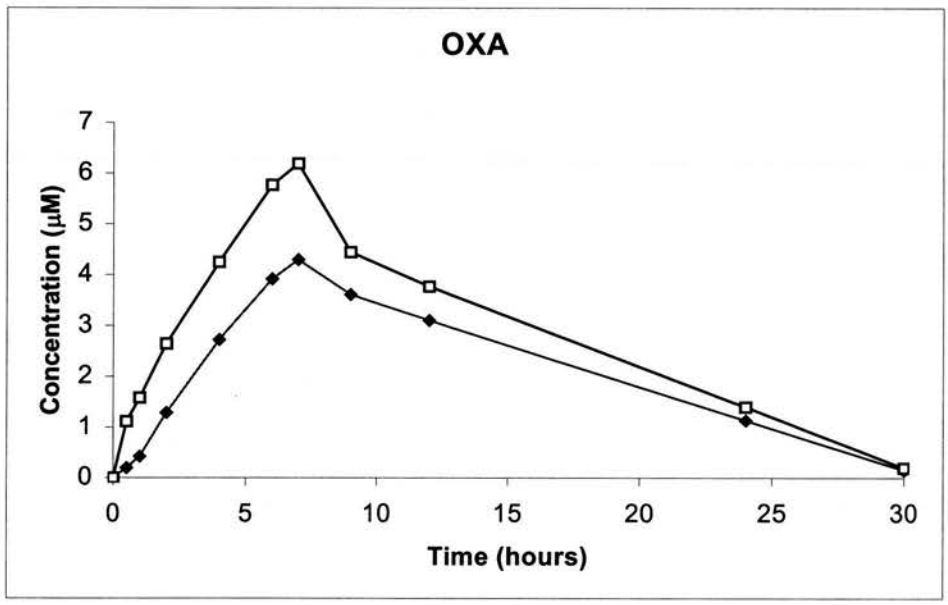


Figure 11.1 d. Mean concentration-time profiles for chloroethylamine and 1, 3 oxazolidine-2-one; □ erythrocytes; ◆ plasma.

Patient	Course	Compound							
		IPA	**IPM**	**CIPA**	**KIPA**	**2-DCEI**	**3-DCEI**	**CEA**	**OXA**
AH	1	1.85	4.09	4.04	-	1.37	1.58	1.29	1.74
	2	1.82	1.43	2.68	3.36	1.00	0.57	1.07	-
DL	1	1.30	1.05	3.41	2.29	1.01	1.00	0.90	1.34
	2	2.13	4.04	4.87	0.93	1.15	1.03	1.87	2.39
AW	1	1.39	2.28	3.39	2.19	1.04	1.00	-	-
	2	1.20	3.22	2.69	1.10	1.27	1.60	1.13	1.09
ML	1	1.01	3.38	1.27	1.31	0.99	1.43	0.67	0.39
VW	1	2.45	4.64	1.90	3.16	1.10	1.05	2.28	1.58
Mean		1.64	3.02	3.03	2.05	1.12	1.16	1.32	1.42
SD		0.50	1.31	1.15	0.98	0.14	0.35	0.57	0.67
n		8	8	8	7	8	8	7	6

Table 11.1. Erythrocyte/plasma concentration ratio at six hours (calculated from the mean curves).

Patient	Course	Compound							
		IPA	**IPM**	**CIPA**	**KIPA**	**2-DCEI**	**3-DCEI**	**CEA**	**OXA**
AH	1	1.17	4.02	2.84	-	1.18	1.10	1.32	1.77
	2	1.53	1.82	4.61	0.68	1.30	2.08	1.32	-
DL	1	1.63	1.45	2.72	1.33	0.44	2.22	1.82	1.09
	2	1.47	3.56	5.09	0.43	0.56	0.32	1.75	2.38
AW	1	1.63	2.49	2.28	2.60	1.36	0.78	-	-
	2	1.20	2.94	2.57	1.12	1.45	1.52	1.05	1.11
ML	1	1.44	2.89	1.46	1.40	0.97	1.06	0.77	0.47
VW	1	2.55	2.16	1.88	2.58	1.35	1.69	3.02	1.43
Mean		1.58	2.67	2.93	1.45	1.08	1.35	1.58	1.37
SD		0.43	0.87	1.27	0.85	0.39	0.65	0.73	0.65
n		8	8	8	7	8	8	7	6

Table 11.2. Ratio of area under the erythrocyte curve / area under the plasma curve (AUC_E/AUC_P).

	IPA	IPM	CIPA	KIPA	2-DCEI	3-DCEI	CEA	OXA
C_6								
p	0.004	0.007	0.004	0.105	0.060	0.124	0.143	0.149
t	3.808	3.420	3.841	1.436	1.808	1.279	1.169	1.141
n	7	7	7	6	7	7	7	7
AUC								
p	0.0003	0.003	0.0002	0.356	0.261	0.418	0.106	0.140
t	5.964	3.859	6.275	0.388	0.675	0.215	1.393	1.211
n	8	8	8	7	8	8	7	6

Table 11.3. Paired t-tests comparing erythrocyte and plasma concentrations at six hours (C_6), and erythrocyte AUC with plasma AUC.

11.4 Discussion

Previous work on the distribution of the oxazaphosphorines and their metabolites has focused on the identification of the circulating transport form of the activated compound, which in effect is responsible for conveying the cytotoxicity of these agents to the tumour cell. Some authors have favoured 4-hydroxycyclophosphamide, in the case of cyclophosphamide (Domeyer and Sladek 1980; Wagner et al 1977), and if correct, this compound should be detectable in the plasma. However, this has not always been the case, a situation explained by the instability of 4-hydroxycyclophosphamide and difficulties in its detection. Nevertheless, some groups have identified persistent, albeit small, amounts of 4-hydroxycyclophosphamide in the plasma of mice (Domeyer and Sladek 1980) and humans (Wagner et al 1977), proposing that low levels of 4-hydroxycyclophosphamide in plasma reflect intracellular trapping of this compound (Domeyer and Sladek 1980).

Other groups have argued that the mustard is the major transport form. Struck et al (1983) studied the activity of IPM against murine tumours, found it comparable to ifosfamide, and postulated that IPM acts by membrane alteration and/or non-DNA alkylation. The authors demonstrated that extracellularly delivered IPM is an effective agent, with antitumour activity and selectivity in vivo comparable to ifosfamide and 4-hydroxyifosfamide.

Sladek has reviewed values of the ratio of CPM AUC to 4-hydroxycyclophosphamide AUC in the plasma of mice, rats and humans, following a dose of cyclophosphamide, and also the sensitivities of mice and human cell cultures to CPM and 4-hydroxycyclophosphamide (Sladek 1988). He was able to calculate percentages of total cell kill hypothetically due to either circulating 4-hydroxycyclophosphamide or CPM, and found that the former compound was the circulating metabolite of major importance in the plasma, although the latter also made a contribution.

However such studies have analysed plasma and have not considered the erythrocyte. The study in this chapter investigated this compartment with a direct technique, avoiding the need for red cell washing or calculation of red cell concentrations from those in plasma and whole blood; the results show that ifosfamide and its metabolites enter the erythrocyte compartment freely, and that IPM and CIPA are concentrated most significantly within this fraction. At the time of the peak erythrocyte IPM concentration in one patient, 82% of the total amount of this compound in whole blood was associated with these cells, 13% was protein bound and 5% was present in plasma water; considering the whole blood AUC for IPM, calculated from the haematocrit, 77% was found to reside within the erythrocyte. In addition to this high uptake by erythrocytes, the rate of loss from these cells is faster than from plasma (figure 11.1 a). As the concentration of IPM in the erythrocyte fraction declines, there is no corresponding increase in CEA levels, indicating that the decline is not due to degradation. In vitro there is a loose association of IPM with the erythrocyte, and this metabolite is released easily into blank plasma (personal communication, E A De Bruijn). Therefore erythrocyte mediated transport of IPM within the circulation is significant, and transfer to tissues plausible. Following intravenous infusion, most of the ifosfamide is carried by erythrocytes and delivered to the liver. It is conceivable that a substantial amount of IPM is transferred from the liver to its site of action associated with the red blood cell and in effect never enters the plasma, a situation similar to the transport of oxygen. It would be instructive to know whether an infusion of IPM itself results in the same distribution pattern in whole blood, as the liver will no longer be a source of IPM.

In early models of the interaction between blood and tissues, compounds have been described as entering tissues from the plasma water phase. However other models propose that inhibition of protein binding occurs on the capillary endothelium releasing protein bound compounds to the tissues (Pardridge and Landaw 1985); as a result tissue levels correspond more closely to the protein bound fraction than to the free fraction. This concept is equally important with regard to erythrocytes, as tissues on the other side of a capillary membrane may be more exposed to compounds accumulated on these cells (Cornford and Landon 1985), and the compounds may

enter tissues just as easily from erythrocytes as from plasma water. Flow through capillaries is intermittent, and red cells are momentarily held in intimate contact with the capillary endothelium, allowing direct exchange between erythrocytes and endothelial cells (Kooyman et al 1995).

The structure and function of the tumour interstitium is significantly different from that of most normal tissues, and solid tumours can possess a special pattern of vascularisation (Jain 1987). For a solid neoplasm to survive, it must develop an adequate blood supply through the process of angiogenesis and then maintain the neovasculature so formed. In achieving this, endothelial cells in tumour vessels can proliferate more than twenty times faster than in normal vessels (Denekamp 1982). The action of cytotoxics on the capillary endothelium may contribute to their anti-tumour effect, as the disruption of a tumour capillary can lead to substantial tumour cell necrosis. Ifosfamide is used particularly in the treatment of solid tumours, and the intimate contact between the capillary endothelium and erythrocytes containing high concentrations of IPM may be important.

The nature of the binding to erythrocytes is currently unclear. Uptake is not restricted to ifosfamide metabolites, and is usually not as high. Many substances bind to erythrocytes (chapter 4); for example, the concentration of bilirubin and cortisol increases in the erythrocyte compartment once albumin binding sites have been saturated, and red cells act as major transporters of acetaldehyde from the liver to tissues (Baraona et al 1987; Hiramatsu and Nisula 1988; Malik et al 1986).

Therefore it must be appreciated that transport by erythrocytes is the rule rather than the exception. As many different compounds can be transported by red cells, usually weakly bound and with a shorter half-life on erythrocytes compared to plasma, a nonspecific binding process such as adsorption to the cell surface comes to mind. The red cell surface area in a human adult is much larger than that of the total capillary endothelium. This ensures that the inner surface of the capillary membrane is in continuous equilbrium with the essentially fragmented membrane of the red cell compartment, which scrapes its surface. However an association of compounds with

proteins in the cell interior, such as that seen during adduct formation between nitrosamine metabolites and globins within haemoglobin (Murphy and Coletta 1993), cannot be excluded. As far as the transfer of compounds is concerned, the relevant factor is whether those associated with a red cell can be released to the capillary endothelium during passage through the capillary.

In conclusion, ifosfamide and its metabolites, especially IPM and CIPA, are sited primarily on the erythrocyte, which may have a role in the transfer of these compounds from the site of their formation to the tissues. Erythrocyte associated IPM then acts as a vital transport form of activated ifosfamide.

PART IV CONCLUSION

Chapter 12

Conclusions and perspectives

Ifosfamide has been used clinically for more than a quarter of a century, and has become firmly established in the treatment of malignant disease. It possesses complex pharmacokinetics, producing metabolites responsible for side effects which are occasionally dose limiting. Dechloroethylation and the production of chloroacetaldehyde is thought to cause neurotoxicity.

The use of oral ifosfamide was restricted by the development of neurotoxicity in all patients treated. Psychometric impairment, manifested by decreased performance in the continuous attention and salford tracker psychometric tests, and EEG abnormalities, with decreased alpha frequency, were seen. The pharmacokinetic studies were performed using a newly developed GC–MS method, and the predicted metabolites CEA and OXA were isolated for the first time in biological fluids. The results indicate that dechloroethylation is important in the development of neurotoxicity, as suggested in the literature, but there was also a tendency to increased CIPA and KIPA formation, at the expense of IPM.

The significance of the red cell compartment in pharmacokinetic studies has been highlighted, with the need to perform a direct analysis on unwashed erythrocytes. IPM and CIPA, the open chain and most polar metabolites, were preferentially located in the red cell compartment, and erythrocyte associated IPM may be an important circulating form of active ifosfamide.

Ifosfamide induced encephalopathy is unusual in two respects. Firstly, it is dependent on structural isomerism, with cyclophosphamide incapable of producing a similar state, after either oral administration, or during the high doses used in autologous peripheral stem cell procedures. The lack of neurotoxicity with cyclophosphamide is thought to result from the relatively low degree of side chain dechloroethylation, but

even so high levels of 3-DCEI have been detected recently, suggesting significant chloroacetaldehyde formation (Momerency et al 1994).

Secondly, the greatly increased incidence after oral doses of ifosfamide, lower than those usually given intravenously. This implies a first pass effect in the liver, or even before this, although studies have shown a bioavailability of approximately 100% (Cerny et al 1986; Kurowski et al 1991; Wagner and Drings 1986). The maximum possible first pass effect of approximately 5%, calculated by Wagner and Drings (1986), should not influence the overall metabolism of ifosfamide, but it is conceivable that during the absorption phase, when high concentrations of ifosfamide reach the liver via the portal vein, a very toxic metabolite responsible for CNS toxicity is generated to a greater extent. However as 20% to 25% of cardiac output flows through the liver, about 1.5 g of a standard intravenous dose (5 g m^{-2}) could be subject to early hepatic metabolism, yet encephalopathy is observed infrequently.

The influence of the gastrointestinal tract on ifosfamide pharmacokinetics and pharmacodynamics is significant. Perhaps the enterohepatic circulation of ifosfamide has an effect, as indicated by rat experiments, where the oral route generates higher biliary chloroacetaldehyde levels compared to the intravenous route (Cerny and Küpfer 1992). There are currently no human data available on this aspect of ifosfamide distribution, nor on the amount of faecal excretion. It is possible that the appearance of ifosfamide within the gastrointestinal tract is responsible for the development of encephalopathy, and that it only occurs in some individuals after intravenous ifosfamide because of variability in the enterohepatic circulation. The metabolism of other drugs within the gut wall, or by the intestinal flora, has been described. One dose of radiation can profoundly change the equilibrium of the intestinal ecosystem, leading to overgrowth of clostridia species (Cuzzolin et al 1992). Data on the enterohepatic circulation of ifosfamide and its metabolites, after intravenous administration, are needed.

Gastric residence time, and gastric and duodenal pH changes, may influence the absorption of ifosfamide. Küpfer et al (1990) noted two distinct compounds,

ifosfamide-I and ifosfamide-II, in aqueous solution. Freshly prepared ifosfamide solutions contain only ifosfamide-II, which is converted to ifosfamide-I on alkalinisation. The rearrangement is fully reversible on acidification. Cyclophosphamide is completely stable under the same conditions.

The mechanism of development of neurotoxicity remains obscure, and the identity of the causative agent elusive. Higher levels of 2-DCEI, 3-DCEI, and particularly CEA, were generated after intravenous administration, and yet no neurotoxicity was observed. The hydrolysis of ifosfamide and its dechloroethyl derivatives, illustrated in figures 10.2 and 10.3, typifies the complexity of ifosfamide breakdown. The phenomenon of encephalopathy remains to be adequately explained, and although chloroacetaldehyde may be contributory, other as yet unidentified metabolites, or processes, could also be important.

The in vivo generation of CEA requires further investigation. It has been suggested that CEA may be produced by ifosfamide decomposition during the analytical derivatisation process, which is solvent dependent (Gilard et al 1997). However the samples obtained following oral and intravenous ifosfamide administration were subjected to the same analytical procedure, and high levels were detected only with the latter.

The mean Cmax of ifosfamide after oral administration, 14 ± 5 $\mu g\ ml^{-1}$ or 53 ± 18 μM, is in keeping with the previously published values of 33 $\mu g\ ml^{-1}$, or 128 μM (Wagner and Drings 1986), and 23 $\mu g\ ml^{-1}$ or 88 μM (Cerny et al 1986), following 1 g of oral ifosfamide. However, the Vss and clearance values of ifosfamide after intravenous administration (mean values of 127 ± 26 L and 12.96 ± 3.81 $L\ hr^{-1}$ respectively) are somewhat higher than those described in the literature. A pharmacokinetic study of a six hour infusion of ifosfamide has not previously been reported, but Granvil et al (1996) found an AUC of 4433 $\mu M\ hr^{-1}$, a clearance of 2.79 $L\ hr^{-1}\ m^{-2}$, and a Vss of 27 $L\ m^{-2}$ after a three hour 3 $g\ m^{-2}$ infusion. Whether the relatively high Vss and clearance values reported in this thesis result from the characteristics of the patient population studied, the co-administration of mitomycin

C, cisplatin or adriamycin, or an underestimate of ifosfamide AUC, requires further clarification.

Other neurophysiological techniques or scanning procedures may shed light on the encephalopathy. Visual evoked potentials provide additional information to the EEG. Positron emission tomography scans can visualise the site of action of drugs. Studies on intracranial blood flow during and after an ifosfamide infusion would be useful, and a suitably labelled ifosfamide preparation may provide more detailed data on pharmacokinetic and pharmacodynamic processes occurring in the CNS.

Methylene blue has been used to reverse and prevent ifosfamide induced encephalopathy. The possibility of using methylene blue with oral ifosfamide arises, but has the disadvantage that ifosfamide would then have to be co-administered with two oral medications, methylene blue and mesna. Further work is required on urinary sarcosine and glutaric acid excretion after ifosfamide administration, and the interaction between ifosfamide and methylene blue.

Another way to surmount the problem of ifosfamide induced encephalopathy is to administer IPM itself. This has not been possible in the past due to the high reactivity of the compound, but a glucose derivative of IPM, D-19575, has recently entered clinical trials. The agent enters cancer cells, releasing the mustard intracellularly, and has the advantage of oral administration (Pohl et al 1995). However one needs to remain cautious. Chloroacetaldehyde has now been shown to be cytotoxic, with activity at least as high as that of 4-hydroxyifosfamide, and it may be necessary for the action of ifosfamide (Brüggemann et al 1997). The pharmacodynamic properties of ifosfamide metabolites warrant further investigation.

Chloroacetaldehyde is known to deplete intracellular glutathione (Lind et al 1989c), and since erythrocyte glutathione levels have been proposed as a predictor of intracellular glutathione concentration (Hercbergs et al 1992), investigating relationships between red cell glutathione levels and ifosfamide neurotoxicity may prove fruitful. Whether the concentrations of ifosfamide and its metabolites in

erythrocytes correlate more closely with ifosfamide toxicity than those in plasma remains to be established. The assay of ifosfamide and its metabolites in the red cell fraction does not indicate the site of these compounds in the erythrocyte, the location of which would be useful in understanding potential delivery mechanisms. Ifosfamide has been found to cause a stomatocytic shape transformation of erythrocytes, indicating an interaction with the lipid bilayer of the cell membrane (Reinhart et al 1999). Red cells have been used to deliver adriamycin (Tonetti et al 1991) and methotexate. Once loaded with methotexate, the erythrocytes can be photosensitized by exposure to haematoporphyrin derivative, and the methotrexate released in a light dependent fashion (Flynn et al 1994). The controllable release of cytotoxics from red cells therefore seems feasible. The finding of higher levels of IPM in the red cell fraction suggests that these cells act as a sink, and using them to deliver IPM may be possible.

Ifosfamide remains a perplexing drug. Although advances have been made in unravelling its complex pharmacokinetics, much is yet to be explained. The need to develop strategies to target the active mustard to the cancer cell, minimising side effects and maximising efficacy, is as relevant now as it was in 1958.

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APPENDIX

Plasma sample (100 μl)	EDTA (μl /ml blood)	Total count (P_C)	Rate of count (P_{CR}) (sec^{-1})	P_{CR} – background (P) (sec^{-1})
1	20	55533	3355	3325
2	20	54982	3322	3292
3	20	53398	3226	3196
4	20	58245	3519	3489
5	20	57129	3452	3422
6	20	56408	3408	3378
7	20	57250	3459	3429
8	20	57610	3481	3451
9	20	57786	3491	3461
10	20	56774	3430	3400

Table A1 a. Calibration data for the MESED tube.

Red cell sample	EDTA (μl /ml blood)	W_1 (g)	W_2 (g)	W_2-W_1 (mg)	Total count (E_C)	Rate of count (E_{CR}) (sec^{-1})	E_{CR} – background (E) (sec^{-1})
1	20	5.1657	5.2779	112.2	4032	243	213
2	20	5.1344	5.2344	100.0	2523	152	122
3	20	5.1202	5.2234	103.2	2613	158	128
4	20	5.1625	5.2642	101.7	2764	167	137
5	20	5.1657	5.2688	103.1	2634	159	129
6	20	5.1587	5.2692	110.5	2758	166	136
7	20	5.1645	5.2670	102.5	2681	162	132
8	20	5.1614	5.2642	102.8	2526	152	122
9	20	5.1180	5.2211	103.1	2694	163	133
10	20	5.1515	5.2570	105.5	2611	158	128

Table A1 b. Calibration data for the MESED tube.

E/P	P_V (E/P x 100) (μl)	P_V /E_V	Trapped P (%)
0.0641	6.41	0.0571	5.71
0.0371	3.71	0.0371	3.71
0.0401	4.01	0.0388	3.88
0.0393	3.93	0.0386	3.86
0.0377	3.77	0.0366	3.66
0.0403	4.03	0.0364	3.64
0.0385	3.85	0.0376	3.76
0.0354	3.54	0.0344	3.44
0.0384	3.84	0.0373	3.73
0.0376	3.76	0.0357	3.57

P_v is the volume of trapped plasma, and E_v is the volume of erythrocytes, assuming the density of erythrocytes is 1.0. The mean percentage trapped plasma is 3.89% (SD ± 0.65) and the median 3.72% (range 3.44 – 5.71).

Table A1 c. Calibration data for the MESED tube.

Patient	Day	CR 1	CR 2	CR 3	CR 4
EG	0	0.367	0.143	0.612	0.113
EG	2	0.341	0.052	0.616	0.057
EG	8	0.348	0.038	0.585	0.050
EG	15	0.338	0.047	0.634	0.155
CH	0	0.343	0.070	1.070	1.203
CH	2	0.387	0.097	0.777	0.152
CH	8	0.385	0.082	0.800	0.184
CH	15	0.394	0.093	0.807	0.136
CB	0	0.447	0.116	0.698	0.250
CB	2	0.527	0.220	0.734	0.211
JW	0	0.338	0.092	0.608	0.100
JW	2	0.355	0.158	0.800	0.417
JW	8	0.342	0.056	0.730	0.247
JW	15	0.349	0.071	0.741	0.063
SO	0	0.452	0.390	0.769	0.372
SO	2	0.458	0.232	1.052	0.497
LR	0	0.287	0.043	0.615	0.043
LR	2	0.307	0.044	0.766	0.255
LR	15	0.400	0.123	0.847	0.219

Table A2. Oral ifosfamide; choice reaction (see table 8.2); reaction time is measured in seconds.

Patient	Day	ST 1	ST 2	ST 3	ST 4	ST 5	ST 6
EG	0	0.307	0.041	0.457	0.023	0.385	0.018
EG	2	0.288	0.030	0.601	0.154	0.391	0.017
EG	8	0.268	0.018	0.462	0.064	0.350	0.019
EG	15	0.296	0.037	0.490	0.029	0.381	0.029
CH	0	0.407	0.045	0.810	0.216	0.547	0.062
CH	2	0.423	0.048	0.852	0.127	0.578	0.050
CH	8	0.402	0.057	0.910	0.285	0.585	0.084
CH	15	0.459	0.033	0.932	0.296	0.614	0.077
CB	0	0.412	0.027	1.050	0.372	0.569	0.061
CB	2	0.508	0.022	0.899	0.223	0.647	0.069
JW	0	0.389	0.040	0.589	0.027	0.487	0.016
JW	2	0.319	0.050	0.628	0.079	0.470	0.027
JW	8	0.325	0.025	1.351	0.665	0.570	0.127
JW	15	0.388	0.056	1.010	0.619	0.555	0.105
SO	0	0.347	0.018	0.627	0.128	0.437	0.034
SO	2	0.293	0.162	0.780	0.198	0.503	0.074
LR	0	0.274	0.040	0.499	0.053	0.378	0.036
LR	2	0.277	0.035	0.619	0.341	0.386	0.048
LR	15	0.294	0.063	1.264	0.723	0.536	0.172

Table A3 a. Oral ifosfamide; salford tracker (see table 8.3); reaction time is measured in seconds.

Patient	Day	ST 7	ST 8	ST 9	ST 10
EG	0	30.80	8.497	7.312	1.393
EG	2	50.20	14.92	7.662	2.230
EG	8	34.60	12.34	7.187	1.475
EG	15	33.80	8.585	8.040	1.682
CH	0	58.20	19.14	11.87	2.325
CH	2	59.80	15.15	12.88	0.782
CH	8	52.40	14.82	13.18	3.963
CH	15	62.40	16.16	15.51	3.012
CB	0	66.00	32.84	10.98	2.076
CB	2	57.00	19.92	8.714	2.092
JW	0	54.40	43.59	7.880	2.604
JW	2	53.00	35.37	8.189	2.295
JW	8	95.40	67.16	14.17	9.048
JW	15	74.00	45.22	12.53	7.269
SO	0	104.4	49.04	12.23	6.392
SO	2	129.6	65.57	19.09	11.19
LR	0	51.80	15.17	9.951	1.691
LR	2	59.80	36.03	10.57	2.405
LR	15	106.2	65.88	16.86	2.899

Table A3 b. Oral ifosfamide; salford tracker (see table 8.3); distance is measured in pixels.

Patient	Day	CA 1	CA 2	CA 3	CA 4
EG	0	0.506	0.082	1	0
EG	2	0.537	0.101	0	0
EG	8	0.535	0.101	0	0
EG	15	0.530	0.122	0	1
CH	0	0.434	0.317	13	2
CH	2	0.574	0.176	11	4
CH	8	0.736	0.099	10	5
CH	15	0.717	0.197	12	1
CB	0	0.589	0.101	5	1
CB	2	0.603	0.094	1	0
JW	0	0.515	0.075	2	1
JW	2	0.522	0.133	1	0
JW	8	0.517	0.072	1	0
JW	15	0.531	0.078	1	0
SO	0	0.542	0.028	14	25
SO	2	0.631	0.261	11	30
LR	0	0.654	0.165	7	1
LR	2	0.740	0.136	5	3
LR	15	0.451	0.485	13	5

Table A4. Oral ifosfamide; continuous attention task (see table 8.4); reaction time is measured in seconds.

Psychometric Test		Day 2	Day 8	Day 15
CR 1	p	0.085	0.332	0.157
	t	1.606	0.506	1.205
	n	6	3	4
CR 3	p	0.242	0.330	0.395
	t	0.754	0.511	0.290
	n	6	3	4
CR 3 – CR 1	p	0.339	0.325	0.481
	t	0.440	0.528	0.053
	n	6	3	4
T 1	p	0.427	0.085	0.180
	t	0.193	2.106	1.077
	n	6	3	4
T 3	p	0.134	0.174	0.068
	t	1.247	1.214	2.025
	n	6	3	4
T 5	p	0.058	0.246	0.059
	t	1.896	0.834	2.177
	n	6	3	4
T 7	p	0.113	0.229	0.094
	t	1.379	0.919	1.695
	n	6	3	4
T 9	p	0.198	0.164	0.026
	t	0.927	1.282	3.106
	n	6	3	4
CA 1	p	0.018	0.183	0.391
	t	2.865	1.158	0.302
	n	6	3	4

Table A5. Comparisons of neurotoxicity on day 1 with days 2, 8 and 15 (t-test).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
JW D1	0.00	0	0	0	0	0	0	0.25	0.032
JW D1	0.48	3.77	0	0.031	0	0	0.09	0.31	0.032
JW D1	1.38	9.14	0.53	0.29	0.19	0	0.15	0.34	0.043
JW D1	2.18	10.45	0.52	0.73	0.23	0	0.33	0.4	0.051
JW D1	4.10	8.52	0.47	0.81	0.32	0.019	0.28	0.43	0.04
JW D1	5.77	8.36	0.45	0.82	0.28	0.014	0.07	0.33	0.034
JW D14	0.00	1.05	0.67	1.04	0	0.089	0.093	0.16	0.042
JW D14	0.63	1.36	1.2	1.25	0.15	0.092	0.12	0.23	0.044
JW D14	1.48	4.78	1.4	1.42	0.17	0.113	0.19	0.37	0.043
JW D14	2.63	9.00	0.77	1.73	0.34	0.172	0.4	0.54	0.081
JW D14	4.97	7.88	0.74	1.98	0.51	0.15	0.36	0.48	0.1
JW D14	7.02	4.58	0.42	1.85	0.42	0.142	0.085	0.24	0.094
NH D1	0.00	0	0	0	0	0.000	0.000	0.197	0.013
NH D1	0.53	4.47	0.163	0.022	0.358	0.024	0.024	0.339	0.039
NH D1	1.60	12.64	0.188	0.521	0.495	0.11	0.084	0.625	0.097
NH D1	2.68	11.11	0.214	0.504	0.609	0.134	0.289	1.138	0.098
NH D1	5.33	10.24	0.522	0.723	0.51	0.106	0.296	0.989	0.08
NH D1	6.98	7.08	0.41	0.672	0.305	0.066	0.141	0.632	0.068
NH D14	0.00	2.07	0.282	1.16	0.274	0.045	0.088	0.238	0.082
NH D14	0.42	4.1	0.359	1.22	0.289	0.088	0.121	0.279	0.087
NH D14	1.30	10.2	-	1.93	0.354	0.227	0.135	0.564	0.1
NH D14	2.05	11.36	0.479	2.34	0.762	0.452	0.195	0.794	0.117
NH D14	4.17	8.93	0.667	2.23	0.648	0.352	0.296	0.893	0.097
NH D14	6.18	7.95	0.547	2.15	0.419	0.136	0.229	0.624	0.073
CH D1	0.00	0	0	0	0	0	0	0.12	0.028
CH D1	0.45	2.19	0	0.01	0	0	0	0.32	0.049
CH D1	1.13	8.87	0.19	0.03	0	0	0.16	0.35	0.057
CH D1	2.10	9.68	0.1	0.18	0.25	0	0.22	0.54	0.073
CH D1	3.37	7.28	0	0.35	0.28	0	0.11	0.46	0.055
CH D1	5.18	5.45	0	0.33	0.27	0.034	0.07	0.29	0.036
CH D14	0.00	1.19	0.33	0.88	0.35	0.088	0	0.2	0.033
CH D14	0.42	2.52	0.55	0.85	0.38	0.1	0	0.32	0.04
CH D14	1.22	8.19	0.71	1.24	0.45	0.11	0.24	0.32	0.033
CH D14	2.30	10.91	0.35	1.28	0.47	0.205	0.33	0.55	0.058
CH D14	3.97	7.07	0.29	1.21	0.3	0.28	0.19	0.49	0.157
CH D14	6.40	5.23	0	1.2	0.24	0.157	0.063	0.29	0.075

Table A6 a. Ifosfamide and ifosfamide metabolite concentrations (μg ml^{-1}) on days 1 (D1) and 14 (D14).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
EG D1	0.00	0	-	0	0	0	-	-	-
EG D1	0.48	21.4	-	0.22	0.1	0.12	-	-	-
EG D1	1.28	20.5	-	0.41	0.32	0.074	-	-	-
EG D1	2.72	13.6	-	0.45	0.24	0.051	-	-	-
EG D1	4.20	8.4	-	0.4	0.18	0.04	-	-	-
EG D1	6.70	5.3	-	0.38	0.053	0.029	-	-	-
EG D14	0.00	4.1	-	0	0	0.04	-	-	-
EG D14	0.53	26.2	-	0.31	0.12	0.19	-	-	-
EG D14	1.20	20.1	-	0.5	0.48	0.096	-	-	-
EG D14	1.95	11.7	-	0.63	0.35	0.072	-	-	-
EG D14	3.95	6.86	-	0.58	0.23	0.048	-	-	-
EG D14	5.78	4.12	-	0.56	0.07	0.024	-	-	-
CB D1	0.00	0	0	0	0	0	0	0.21	0.076
CB D1	0.42	7.21	0.07	0.23	0.036	0	0.19	0.35	0.08
CB D1	1.13	11.7	0.38	0.75	0.136	0	0.2	0.53	0.16
CB D1	2.30	14.4	0.36	0.81	0.46	0	0.39	0.32	0.083
CB D1	4.88	9	0.21	0.85	0.22	0.02	0.22	0.27	0.074
CB D1	6.63	7.85	0.1	0.76	0.14	0.04	0.18	0.19	0.07
LR D1	0.00	0	0	0	0	0	0	0.03	0.021
LR D1	0.52	0.39	0.034	0.021	0.009	0.021	0.023	0.248	0.097
LR D1	1.33	4.5	0.068	0.081	0.095	0.021	0.131	0.36	0.098
LR D1	2.28	7.22	0.357	0.506	0.214	0.177	0.216	1.016	0.096
LR D1	4.22	8.01	0.433	1.34	0.706	0.347	0.224	0.834	0.108
LR D1	6.15	6.38	0.4	1.26	0.52	0.276	0.18	0.389	0.076
JT D1	0.00	0	0	0	0	0	0	0.209	0.067
JT D1	0.60	8.32	0.072	0.133	0.031	0.015	0.067	0.254	0.118
JT D1	1.47	8.58	0.269	0.156	0.428	0.073	0.09	0.476	0.118
JT D1	2.70	8.42	0.442	0.563	0.896	0.135	0.084	0.603	0.102
JT D1	4.55	7.77	0.316	0.646	0.583	0.207	0.047	0.634	0.077
JT D1	7.38	4.41	0.101	0.602	0.237	0.149	0.039	0.334	0.072
JT D14	0.00	1.25	0.159	0.242	0.035	0.008	0	0.127	0.049
JT D14	0.55	2.16	0.191	0.47	0.233	0.026	0.01	0.382	0.057
JT D14	1.22	7.17	0.197	0.5	0.428	0.067	0.06	0.75	0.078
JT D14	2.58	5.31	0.322	0.454	0.895	0.07	0.054	1.01	0.137
JT D14	5.52	3.96	0.227	0.439	1.09	0.075	0.055	0.771	0.118
JT D14	7.35	3	0.167	0.393	0.7	0.049	0.045	0.506	0.106

Table A6 b. Ifosfamide and ifosfamide metabolite concentrations ($\mu g\ ml^{-1}$) on days 1 (D1) and 14 (D14).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
SO D1	0.00	0	0	0	0	0	0	0.078	0.066
SO D1	0.48	7.22	0.026	0.154	0.041	0.071	0.112	0.226	0.077
SO D1	1.47	16.39	0.077	0.373	0.125	0.099	0.122	0.689	0.094
SO D1	3.13	17.14	0.28	0.693	0.453	0.147	0.189	0.974	0.143
SO D1	5.12	13.29	0.357	0.889	0.339	0.089	0.198	0.896	0.099
SO D1	6.73	9.82	0.325	0.916	0.168	0.075	0.074	0.759	0.076
PC D1	0.00	0	0	0	0	0	0	0.135	0.016
PC D1	0.45	4.51	0.24	0.35	0.064	0.03	0	0.298	0.017
PC D1	1.15	10.35	0.52	0.36	0.062	0.04	0.18	0.315	0.024
PC D1	2.32	12.06	0.54	1.79	0.098	0.38	0.25	0.501	0.058
PC D1	4.15	10.86	0.45	1.91	0.076	0.41	0.26	0.258	0.046
PC D1	6.32	6.12	0.16	1.73	0.064	0.22	0.18	0.236	0.029
PC D14	0.00	0	0	0.06	0	0	0.02	0.179	0.022
PC D14	0.57	-	-	-	-	-	-	0.379	0.021
PC D14	1.42	6.48	0.76	0.69	0.064	0.071	0.17	0.428	0.019
PC D14	2.40	9.54	0.84	1.92	0.136	0.356	0.22	0.493	0.027
PC D14	4.47	5.94	0.42	2.16	0.122	0.452	0.24	0.426	0.039
PC D14	6.70	5.04	0.23	1.75	0.102	0.361	0.2	0.34	0.03
LJ D1	0.00	0	0	0	0	0	0	-	-
LJ D1	0.08	0	0	0.02	0	0.056	0	-	-
LJ D1	1.08	1.82	0	0.04	0.02	0.073	0.11	-	-
LJ D1	2.08	16.48	0.46	1.09	0.38	0.101	0.28	-	-
LJ D1	4.08	10.94	0.28	1.02	0.25	0.21	0.29	-	-
LJ D1	5.92	7.6	0.24	0.83	0.21	0.103	0.2	-	-
RC D1	0.00	0	0	0	0	0	0	0.062	0.042
RC D1	0.48	9.42	0	0.019	0	0	0.022	0.503	0.048
RC D1	1.17	12.48	0	0.062	0.039	0.014	0.049	0.608	0.063
RC D1	2.13	21.71	0.089	0.121	0.125	0.107	0.159	0.757	0.085
RC D1	4.80	11.33	0.185	0.5	0.175	0.085	0.161	0.981	0.084
RC D1	6.80	8.42	0	0.469	0.113	0.058	0.033	0.599	0.077
RC D14	0.00	5.02	0.388	1.15	0.272	0.068	0.021	0.503	0.051
RC D14	0.60	13.94	1.19	1.39	0.634	0.123	0.084	0.882	0.062
RC D14	1.05	18.45	1.01	1.51	0.837	0.193	0.146	0.978	0.093
RC D14	3.30	13.59	0.95	1.91	0.93	0.368	0.118	0.831	0.102
RC D14	4.97	8.52	0.75	1.92	0.623	0.271	0.027	0.664	0.051

Table A6 c. Ifosfamide and ifosfamide metabolite concentrations ($\mu g\ ml^{-1}$) on days 1 (D1) and 14 (D14).

Patient	Age (years)	Sex	Diagnosis	Tmax (hr)	Cmax (μM)	L_z (hr^{-1})	$T_{1/2}$ (hr)	AUC_t (μM . hr)	AUC_8 (μM . hr)	KA (hr^{-1})
CB	45	M	NSCLC	2.30	55.2	0.143	4.84	260	312	0.87
EG	54	F	Cervical Ca	0.48	82.2	0.184	3.76	302	338	4.17
CH	58	F	Cervical Ca	2.15	37.1	0.160	4.33	136	231	0.95
JW	59	F	Cervical Ca	2.18	40.1	0.063	10.96	179	265	0.92
LR	43	F	Cervical Ca	4.22	30.7	0.118	5.88	139	198	0.47
SO	48	F	Cervical Ca	3.13	65.7	0.188	3.69	344	407	0.64
PC	60	M	NSCLC	2.32	46.2	0.264	2.62	223	286	0.86
RC	58	M	NSCLC	2.13	83.1	0.148	4.67	339	387	0.94
JT	63	M	NSCLC	1.47	32.9	0.200	3.46	199	212	1.36
NH	33	F	Cervical Ca	1.60	48.4	0.224	3.10	251	287	1.25
LJ	70	F	Cervical Ca	2.08	63.1	0.198	3.50	207	301	0.96

Table A7. Oral ifosfamide; ifosfamide pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)	L_z (hr^{-1})	$T_{1/2}$ (hr)
CB	-	-	-	-	-	-	-
EG	0.53	100.4	15.7	242	309	0.279	2.49
CH	2.30	41.8	4.6	172	214	0.124	5.59
JW	2.63	34.5	4.0	167	190	0.265	2.62
LR	-	-	-	-	-	-	-
SO	-	-	-	-	-	-	-
PC	2.40	36.5	0	155	185	0.074	9.41
RC	1.05	70.7	19.2	256	489	0.280	2.48
JT	1.22	27.5	4.8	124	133	0.152	4.57
NH	2.05	43.5	7.9	207	272	0.058	11.98
LJ	-	-	-	-	-	-	-

Table A8. Oral ifosfamide; ifosfamide pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	1.13	1.91	8.01	11.17
EG	-	-	-	-
CH	1.13	0.96	1.31	-
JW	1.38	2.67	11.99	22.64
LR	4.22	2.18	9.17	17.50
SO	5.12	1.80	7.76	12.96
PC	2.32	2.72	12.34	17.25
RC	4.80	0.93	2.97	2.97
JT	2.70	2.23	9.22	10.84
NH	5.33	2.63	11.03	15.69
LJ	2.08	2.32	7.20	14.59

Table A9. Oral ifosfamide; 2-DCEI pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	-	-	-	-	-
CH	1.22	3.61	1.66	10.68	26.39
JW	1.48	7.05	3.37	29.36	33.96
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	2.40	4.23	0	16.52	20.96
RC	0.60	5.99	1.95	23.06	45.61
JT	2.58	1.62	0.80	8.76	10.84
NH	4.17	3.36	1.42	16.37	26.19
LJ	-	-	-	-	-

Table A10. Oral ifosfamide; 2-DCEI pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	4.88	4.28	24.47	33.36
EG	2.72	2.27	12.73	18.47
CH	3.37	1.76	5.38	17.00
JW	-	-	17.12	31.88
LR	4.22	6.75	23.29	40.13
SO	-	-	21.20	30.20
PC	4.15	9.62	44.90	64.62
RC	4.80	2.52	9.66	15.51
JT	4.55	3.25	17.58	21.03
NH	5.33	3.64	18.26	24.33
LJ	2.08	5.49	22.16	37.29

Table A11 Oral ifosfamide; 3-DCEI pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	1.95	3.17	0.00	15.26	27.15
CH	2.32	6.47	4.43	38.11	51.83
JW	4.97	9.97	5.24	60.08	71.90
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	4.47	10.88	0.30	52.26	68.11
RC	-	-	5.79	42.60	79.32
JT	1.22	2.52	1.22	16.33	19.24
NH	2.05	11.78	5.84	64.12	88.92
LJ	-	-	-	-	-

Table A12 Oral ifosfamide; 3-DCEI pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	2.30	1.57	5.36	7.93
EG	1.28	1.09	3.96	5.58
CH	3.37	0.96	3.24	10.54
JW	4.10	1.09	4.37	10.10
LR	4.22	2.41	7.71	14.47
SO	3.13	1.55	5.97	8.48
PC	2.32	0.33	1.57	4.50
RC	4.80	0.60	2.66	4.84
JT	2.70	3.06	11.81	13.28
NH	2.68	2.08	11.19	13.92
LJ	2.08	1.30	4.27	8.99

Table A13. Oral ifosfamide; CIPA pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	1.20	1.66	0.00	4.64	7.78
CH	2.30	1.60	1.19	7.75	11.62
JW	4.97	1.74	0.00	8.26	11.32
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	2.40	0.46	0.00	2.25	4.75
RC	3.30	3.17	0.93	13.20	29.03
JT	5.52	3.72	0.12	19.52	22.28
NH	2.05	2.60	0.93	11.50	17.45
LJ	-	-	-	-	-

Table A14. Oral ifosfamide; CIPA pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	0.29	4.69
EG	0.48	0.44	1.27	3.43
CH	-	-	0.28	-
JW	4.10	0.07	0.18	3.05
LR	4.22	1.26	4.44	9.45
SO	3.13	0.53	2.44	4.85
PC	4.15	1.49	6.03	10.10
RC	2.13	0.39	1.67	3.72
JT	4.55	0.75	3.60	5.01
NH	2.68	0.49	2.44	4.15
LJ	4.08	0.76	2.69	6.12

Table A15. Oral ifosfamide; KIPA pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	0.54	0.73	0.15	1.45	3.64
CH	3.97	1.03	0.32	4.40	7.72
JW	2.63	0.63	0.32	3.56	5.82
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	4.47	1.64	0.00	7.27	11.29
RC	3.30	1.34	0.25	4.69	13.05
JT	5.52	0.27	0.03	1.67	2.82
NH	2.05	1.64	0.16	6.29	9.82
LJ	-	-	-	-	-

Table A16. Oral ifosfamide; KIPA pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	2.30	1.76	7.42	11.37
EG	-	-	-	-
CH	2.10	1.00	2.71	7.68
JW	2.18	1.49	5.25	10.54
LR	4.22	1.01	4.75	10.00
SO	5.12	0.90	4.48	6.73
PC	4.15	1.18	5.66	10.33
RC	-	-	3.26	4.77
JT	1.47	0.41	1.95	3.41
NH	-	-	6.24	8.73
LJ	4.08	1.31	5.75	11.70

Table A17. Oral ifosfamide; IPM pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	-	-	-	-	-
CH	2.30	1.52	0.00	5.02	7.72
JW	2.63	1.81	0.42	8.23	10.10
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	4.47	1.09	0.09	5.84	9.79
RC	1.05	0.66	0.10	2.17	8.02
JT	5.52	0.25	0.00	1.58	3.09
NH	4.17	1.34	0.40	5.97	11.61
LJ	-	-	-	-	-

Table A18. Oral ifosfamide; IPM pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	1.13	4.02	8.68	19.44
EG	-	-	-	-
CH	2.10	5.28	17.98	54.29
JW	4.10	2.26	8.80	40.88
LR	2.28	12.40	46.53	71.50
SO	3.13	11.27	57.47	78.39
PC	2.32	4.60	14.21	29.09
RC	4.80	11.56	58.10	76.04
JT	4.55	5.34	25.28	31.66
NH	2.68	11.83	54.70	70.87
LJ	-	-	-	-

Table A19. Oral ifosfamide; CEA pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	-	-	-	-	-
CH	2.30	5.41	1.01	23.26	35.00
JW	2.63	3.65	0.00	20.75	25.84
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	2.40	4.50	0.55	23.26	34.04
RC	1.05	11.52	5.55	47.79	95.77
JT	2.58	10.07	0.00	56.97	64.09
NH	4.17	8.75	0.52	38.48	61.04
LJ	-	-	-	-	-

Table A20. Oral ifosfamide; CEA pharmacokinetic parameters day 14.

Patient	Tmax (hr)	Cmax (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	1.13	0.96	0.92	9.69
EG	-	-	-	-
CH	2.10	0.52	1.49	11.68
JW	2.18	0.22	0.57	8.13
LR	4.22	1.00	5.17	14.52
SO	3.13	0.88	2.76	8.44
PC	2.32	0.48	1.61	7.73
RC	2.13	0.49	2.64	10.00
JT	0.60	0.59	1.95	5.61
NH	2.68	0.98	5.28	11.37
LJ	-	-	-	-

Table A21. Oral ifosfamide; OXA pharmacokinetic parameters day 1.

Patient	Tmax (hr)	Cmax (μM)	Cmin (μM)	AUC_t (μM . hr)	AUC_8 (μM . hr)
CB	-	-	-	-	-
EG	-	-	-	-	-
CH	3.97	1.48	0.06	4.13	10.54
JW	4.97	0.78	0.11	3.68	9.79
LR	-	-	-	-	-
SO	-	-	-	-	-
PC	4.47	0.26	0.07	1.03	6.90
RC	3.30	0.69	0.10	2.18	12.53
JT	2.58	0.80	0.00	5.04	8.99
NH	2.05	1.19	0.79	5.86	15.26
LJ	-	-	-	-	-

Table A22. Oral ifosfamide; OXA pharmacokinetic parameters day 14.

Patient	Compound							
	IPA	**2-DCEI**	**3-DCEI**	**CIPA**	**KIPA**	**IPM**	**CEA**	**OXA**
CB	-	-	-	-	-	-	-	-
EG	1.22	-	1.4	1.52	1.67	-	-	-
CH	1.13	3.77	3.67	1.68	-	1.52	1.02	2.87
JW	0.86	2.64	-	1.59	9.05	1.21	1.61	3.58
LR	-	-	-	-	-	-	-	-
SO	-	-	-	-	-	-	-	-
PC	0.79	1.56	1.13	1.39	1.10	0.92	0.98	0.55
RC	0.85	6.43	-	5.31	3.44	-	1.00	1.40
JT	0.84	0.73	0.77	1.22	0.36	0.61	1.88	1.37
NH	0.90	1.28	3.24	1.25	3.37	-	0.74	1.22
LJ	-	-	-	-	-	-	-	-
Mean	0.94*	2.73	2.04	1.99	3.17	1.07	1.21	1.83
SD	0.16*	2.11	1.32	1.47	3.13	0.39	0.44	1.14
n	7*	6	5	7	6	4	6	6

* excluding PC, mean = 0.97, SD = 0.17, and n = 6

Table A23. Oral ifosfamide; ratio of day 14 Cmax / day 1 Cmax.

Compound	r	p	n
IPA			
D 1 and D 2	0.0436	0.4673	6
D 14 and D 15	-0.2280	0.3862	4
2-DCEI			
D 1 and D 2	0.2236	0.3588	5
D 14 and D 15	0.7298	0.2396	3
3-DCEI			
D 1 and D 2	-0.2880	0.3561	4
D 14 and D 15	-0.2350	0.3826	4
CIPA			
D 1 and D 2	-0.4020	0.2146	6
D 14 and D 15	-0.8300	0.0850	4
KIPA			
D 1 and D 2	-0.7160	0.1421	4
D 14 and D 15	-0.8410	0.0795	4
IPM			
D 1 and D 2	0.9547	0.0058	5
D 14 and D 15	0.9077	0.1378	3
CEA			
D 1 and D 2	-0.8850	0.0232	5
D 14 and D 15	-0.9940	0.0358	3
OXA			
D 1 and D 2	-0.3110	0.3050	5
D 14 and D 15	-0.3310	0.3925	3

Table A24. Day 1 (D1) Cmax and day 2 (D2) α frequency (percentage change), and day 14 (D14) Cmax and day 15 (D15) α frequency (percentage change), correlations for ifosfamide and its metabolites; (r = Pearsons correlation coefficient).

	r	p	n
IPA	0.0094	0.4953	4
2-DCEI	0.7621	0.2242	3
3-DCEI	0.0296	0.4906	3
CIPA	0.8951	0.0524	4
KIPA	0.7520	0.2291	3
CEA	0.8811	0.1568	3
OXA	0.9980	0.0203	3

Table A25. Correlation of day 14 Cmax / day 1 Cmax ratio with day 15 α frequency, (percentage change), for ifosfamide and its metabolites; (r = Pearsons correlation coefficient).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
AH	0.00	0	0	0	0	0	0	0.47	0.08
Cycle 1	0.15	8.02	0	0.02	0.2	0.04	0.05	1.67	0.42
Plasma	1.05	11.87	0.1	0.2	0.22	0.07	0.24	2.8	0.52
	2.07	19.95	0.22	0.6	0.52	0.07	0.44	3.47	0.57
	4.15	24.98	0.62	1.81	0.85	0.11	0.88	6.07	0.81
	6.27	29.3	1.03	2.31	1.09	0.14	0.93	14.7	1.2
	7.48	28.32	1.25	2.41	1.52	0.16	0.82	8.6	0.52
	11.83	25.45	1.6	2.54	1.71	0.18	0.77	3.91	0.3
	21.92	15.02	0.76	2.01	0.85	0.12	0.31	2.24	0.15
	25.45	11.73	0.58	1.79	0.56	0.08	0.32	1.43	0.11
$T_{1/2}$		9.9							
CL		7.69							
Vss		119							
AH	0.00	0	0	0	0	-	0	0	0
Cycle 1	0.15	8.031	0.161	0.104	0.286	-	0.168	1.22	0.098
Erythrocyte	1.05	15.354	0.314	0.361	0.413	-	0.476	2.95	0.509
	2.07	29.577	0.617	1.125	0.606	-	1.255	3.824	0.934
	4.15	42.207	0.877	2.625	2.683	-	2.424	8.932	1.71
	6.27	54.814	1.384	3.661	4.435	-	3.964	18.624	2.047
	7.48	52.408	1.507	4.104	5.09	-	4.572	11.73	1.292
	11.83	38.593	1.943	4.48	5.902	-	4.254	5.786	0.643
	21.92	15.254	0.942	3.339	2.565	-	1.596	2.476	0.375
	25.45	8.396	0.666	2.508	1.021	-	1.038	1.677	0.11

Table A26 a. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
AH	0.00	0	0	0	0	0	0	0.01	0
Cycle 2	0.33	4.24	0	0.05	0.02	0	0.06	0.23	0
Plasma	1.27	10.35	0	0.11	0.11	0	0.19	0.84	0
	2.53	23.6	0.09	0.62	0.15	0	0.33	1.37	0
	4.45	26.2	0.31	1.89	0.41	0.004	0.37	2.18	0.02
	6.32	29.3	0.84	3.07	0.94	0.01	0.62	3.23	0.06
	7.48	32.1	1.02	3.14	1.51	0.02	1.07	3.79	0.07
	8.88	29.9	0.84	3.24	1.12	0.04	0.76	2.08	0.02
	14.72	14.9	0.65	3.32	0.86	0.06	0.4	0.87	0
	20.53	10.05	0.32	1.97	0.19	0.04	0.36	0.54	0
	24.07	7.58	0	1.32	0.11	0.008	0.24	0.36	0
$T_{1/2}$		8.7							
CL		10.07							
Vss		120							
AH	0.00	0	0	0	0	0	0	0.02	0
Cycle 2	0.33	6.682	0	0.04	0.03	0	0.226	0.573	0
Erythrocyte	1.27	15.701	0	0.339	0.193	0	0.46	0.913	0
	2.53	48.12	0.09	0.662	0.607	0.008	0.517	0.996	0
	4.45	52.071	0.383	1.028	1.158	0.021	0.754	2.907	0
	6.32	51.638	0.85	1.771	2.415	0.031	0.838	3.355	0
	7.48	43.425	1.332	2.34	3.827	0.03	1.756	3.78	0
	8.88	37.9	1.224	5.38	3.873	0.019	1.072	2.745	0
	14.72	-	-	-	-	-	-	-	-
	20.53	25.791	0.445	4.006	2.663	0.009	0.817	1.111	0
	24.07	9.242	0.125	3.294	1.481	0.009	0.479	0.568	0

Table A26 b. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
DL	0.00	0	0	0	0	0	0	0.05	0
Cycle 1	0.23	4.17	0	0.02	0	0	0	0.66	0
Plasma	0.88	12.4	0	0.3	0.095	0	0.13	0.94	0.058
	2.42	16.9	0.13	0.41	0.56	0.26	0.26	1.95	0.12
	4.20	28.6	0.46	1.53	1.26	0.36	0.63	2.93	0.15
	6.38	50.2	0.52	2.5	2.38	0.43	0.96	3.82	0.091
	7.75	39.2	0.68	2.28	2.79	0.56	0.9	2.23	0.063
	17.22	11.2	0.22	1.56	0.92	0.15	0.28	-	-
	21.48	6.91	0.08	1.36	0.73	0.14	0.19	0.55	0
$T_{1/2}$		6.1							
CL		11.02							
Vss		93							
DL	0.00	0	0	0	0	0	0	0.2	0
Cycle 1	0.23	5.767	0	0.192	0.107	0	0.107	0.949	0
Erythrocyte	0.88	17.653	0	0.246	0.25	0	0.516	-	-
	2.42	30.193	0.151	0.378	1.268	0	0.517	1.2	0
	4.20	47.371	0.503	1.412	3.308	0.317	0.737	2.137	0.139
	6.38	63.193	0.52	2.564	8.544	1.084	0.992	3.627	0.133
	7.75	41.698	0.251	2.441	9.351	1.257	1.608	1.93	0.028
	17.22	8.338	0.016	0.981	2.882	0.482	0.398	1.41	0.02
	21.48	7.542	0.005	0.953	1.995	0.376	0.254	-	-

Table A26 c. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
DL	0.00	0	0	0	0	0	0	0.04	0
Cycle 2	0.25	3.63	0	0	0	0	0	0.33	0
Plasma	1.05	9.63	0	0.34	0.04	0.25	0.04	0.79	0.005
	2.90	16.7	0.13	1.58	0.07	0.29	0.13	1.59	0.024
	4.70	21.8	0.45	2.49	0.25	0.59	0.23	2.21	0.068
	6.35	22.4	0.74	6.49	0.45	0.94	0.27	3.5	0.073
	8.27	27.8	1.02	5.61	0.59	1.72	0.41	2.88	0.069
	17.50	12	0.59	4.11	0.21	0.86	0.09	1.02	0
	21.58	6.47	0.3	3.89	0.13	0.73	0.06	0.72	0
$T_{1/2}$		4.6							
CL		14.42							
Vss		126							
DL	0.00	0	0	0	0	0	0	0.426	0
Cycle 2	0.25	-	-	-	-	-	-	0.877	0
Erythrocyte	1.05	19.353	0	0.158	0.083	0	0.34	1.444	0
	2.90	33.53	0.398	1.484	0.477	0	0.602	3.048	0.041
	4.70	34.417	0.546	3.026	1.524	0.59	1.034	4.193	0.102
	6.35	50.647	0.847	6.876	2.004	0.854	1.053	6.812	0.198
	8.27	48.393	0.623	5.556	2.541	1.248	1.289	5.099	0.177
	17.50	12.247	0.226	4.946	1.046	0.635	0.304	2.146	0.032
	21.58	5.57	0	3.182	0.687	0.344	0.199	1.235	0

Table A26 d. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
AW	0.00	0	0	0	0	0	0	0	0
Cycle 1	0.57	3.7	0	0.09	0.18	0	0.08	2.01	0
Plasma	1.28	11.2	0.09	0.28	0.4	0	0.41	7.32	0.04
	3.05	21.9	0.12	0.56	0.58	0	1.31	11.2	0.14
	4.65	28	0.36	1.77	0.74	0.02	1.55	14	0.26
	6.83	29.1	0.67	3.41	0.83	0.047	1.6	15.2	0.3
	9.20	20.7	0.65	4.34	1.04	0.111	0.72	11.6	0.19
	16.53	8.8	0.29	2.98	0.49	0.043	0.34	5.1	0.11
	20.58	5.41	0.21	2.5	0.32	0.029	0.24	3.21	0.05
	24.62	3.45	0.15	2.23	0.28	0.011	0.12	1.2	0
$T_{1/2}$		6.2							
CL		12.48							
Vss		100							
AW	0.00	0	0	0	0	0	0	-	-
Cycle 1	0.57	5.362	0.083	0.371	0.658	0	0.194	-	-
Erythrocyte	1.28	12.862	0.132	0.415	1.377	0	1.085	-	-
	3.05	27.614	0.203	0.913	1.661	0.021	3.232	-	-
	4.65	40.884	0.464	1.936	2.319	0.029	4.324	-	-
	6.83	-	-	-	-	-	-	-	-
	9.20	37.532	0.816	4.901	3.534	0.193	1.936	-	-
	16.53	19.398	0.456	3.635	1.082	0.073	0.787	-	-
	20.58	10.179	0.366	3.155	0.881	0.051	0.531	-	-
	24.62	5.809	0.244	2.417	0.664	0.042	0.338	-	-

Table A26 e. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
AW	0.00	0	0	0	0	0	0	0	0
Cycle 2	0.67	6.61	0.05	0.15	0.14	0.14	0.13	4.53	0
Plasma	1.32	13.1	0.08	0.24	0.36	0.18	0.38	11.1	0.024
	2.95	14.7	0.28	1.02	0.72	0.2	0.5	13.8	0.036
	4.75	16.3	0.47	1.46	1.04	0.29	0.64	16.2	0.041
	6.87	21.3	0.64	2.54	1.78	0.33	0.84	19.9	0.054
	8.25	19.2	0.58	2.57	2.1	0.39	0.99	17.5	0.038
	11.63	13.5	0.38	2.32	1.82	0.24	0.71	11.9	0.025
	15.03	10.9	0.22	2.12	1.44	0.14	0.46	8.65	0.011
$T_{1/2}$		11.0							
CL		11.81							
Vss		175							
AW	0.00	0	0	0	0	0	0	0	0
Cycle 2	0.67	7.13	0.06	0.181	0.389	0.15	0.286	5.237	0.011
Erythrocyte	1.32	14.243	0.111	0.365	0.921	0.211	0.837	12.347	0.03
	2.95	17.713	0.519	1.477	1.738	0.242	1.549	15.151	0.04
	4.75	20.04	0.699	3.07	2.827	0.321	2.063	18.382	0.046
	6.87	24.937	0.744	3.558	4.689	0.361	2.721	22.497	0.058
	8.25	23.564	0.902	3.941	5.882	0.411	3.058	19.058	0.041
	11.63	17.76	0.588	3.567	4.937	0.282	2.466	16.575	0.026
	15.03	14.017	0.324	3.253	3.83	0.161	1.395	10.157	0.012

Table A26 f. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
ML	0.00	0	0	0	0	0	0	0	0
Cycle 1	0.55	2.72	0.17	0.19	0.09	0.02	0.019	0.45	0.019
Plasma	1.52	7.08	0.35	0.38	0.45	0.026	0.13	1.23	0.058
	2.63	9.37	0.52	0.56	1.44	0.037	0.39	3.61	0.176
	4.53	15.67	0.92	1.46	2.48	0.066	0.42	6.07	0.254
	6.75	20.37	1.02	3.02	3.33	0.12	0.63	9.73	0.367
	9.08	16.95	0.73	3.6	3.59	0.061	0.81	8.62	0.302
	11.10	14.65	0.53	4	2.75	0.068	0.49	7.02	0.274
	31.03	0.47	0.052	2.33	0.07	0.011	0.14	0.21	0
$T_{1/2}$		4.0							
CL		19.70							
Vss		137							
ML	0.00	0	0	0	0	0	0	0	0
Cycle 1	0.55	4.478	0.224	0.297	0.304	0.031	0.213	3.473	0.106
Erythrocyte	1.52	13.48	0.597	0.616	0.825	0.095	0.591	6.226	0.2
	2.63	23.381	0.767	0.914	2.298	0.158	0.905	9.838	0.287
	4.53	38.729	0.92	1.824	4.549	0.263	1.653	14.753	0.421
	6.75	49.432	1.159	3.041	6.353	0.345	3.117	21.844	0.574
	9.08	42.624	0.923	4.19	7.385	0.253	2.439	12.908	0.396
	11.10	30.108	0.723	4.536	4.197	0.124	1.026	10.375	0.355
	31.03	2.485	0	3.209	0.22	0.021	0.119	3.351	0

Table A26 g. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites (μg ml^{-1}); plasma ifosfamide $T_{1/2}$ (hr), CL (L hr^{-1}) and Vss (L).

Patient	Time (hr)	IPA	2-DCEI	3-DCEI	CIPA	KIPA	IPM	CEA	OXA
VW	0.00	0	0	0	0	0	0	0.12	0
Cycle 1	0.33	0.87	0.12	0.33	0.09	0.06	0.32	1.79	0
Plasma	1.58	8.41	0.71	1.22	0.36	0.09	0.4	3.85	0
	2.78	15	1.25	2.86	0.98	0.12	0.72	8.54	0.13
	4.68	17.4	2.87	4.6	1.56	0.32	0.82	8.86	0.15
	6.67	22.4	2.97	6.23	2.02	0.44	1.09	13.4	0.25
	7.97	29.1	3.56	9.91	2.95	0.5	0.98	10.1	0.2
	16.87	6.1	1.07	5.36	1.7	0.34	0.43	6.29	0.11
	21.62	4.28	0.89	2.15	0.56	0.27	0.31	3.7	0
	26.02	2.69	0.69	1.3	0.09	0.06	0.19	2.37	0
$T_{1/2}$		6.6							
CL		16.51							
Vss		147							
VW	0.00	0	0	0	0	0	0	0.216	0
Cycle 1	0.33	1.234	0.334	0.962	0.133	0.264	0.524	3.034	0
Erythrocyte	1.58	9.428	1.364	2.142	0.424	0.326	1.118	6.616	0
	2.78	16.394	1.711	2.303	1.334	0.377	2.103	7.768	0
	4.68	19.544	2.881	6.036	2.289	0.524	2.868	8.067	0.075
	6.67	21.971	2.906	8.942	2.417	0.526	3.609	7.997	0.089
	7.97	30.279	3.271	8.516	3.593	0.596	3.574	9.285	0.114
	16.87	11.246	1.359	5.221	2.826	0.372	1.062	4.232	0.056
	21.62	6.467	0.729	3.008	0.978	0.291	0.653	1.878	0
	26.02	5.081	0.551	1.729	0.262	0.146	0.404	1.287	0

Table A26 h. Plasma and erythrocyte concentrations of ifosfamide and ifosfamide metabolites ($\mu g\ ml^{-1}$); plasma ifosfamide $T_{1/2}$ (hr), CL ($L\ hr^{-1}$) and Vss (L).

Patient	IPA		2-DCEI		3-DCEI		CIPA	
	P	E	P	E	P	E	P	E
AH1	2665	3128	160	189	529	580	110	313
AH2	2034	3111	58	75	339	703	51	233
DL1	2015	3278	37	16	385	853	145	396
DL2	1541	2266	71	39	1883	604	24	121
AW1	1436	2335	51	69	723	564	78	177
AW2	1518	1819	35	51	539	817	141	363
ML1	914	2327	61	83	859	1450	139	262
VW1	1225	1769	273	266	671	714	125	182

Table A27 a. Plasma (P) and erythrocyte (E) AUCs for ifosfamide and ifosfamide metabolites (μM . hr).

Patient	KIPA		IPM		CEA		OXA	
	P	E	P	E	P	E	P	E
AH1	15	-	86	347	1570	2066	128	228
AH2	3	2	59	106	444	585	3	-
DL1	53	70	55	80	509	926	14	15
DL2	138	59	20	71	571	1000	8	19
AW1	4	10	74	184	2408	-	36	-
AW2	17	19	60	175	3695	3863	6	6
ML1	5	14	59	128	1350	4081	62	88
VW1	29	40	74	214	2471	1890	30	14

Table A27 b. Plasma (P) and erythrocyte (E) AUCs for ifosfamide and ifosfamide metabolites (μM . hr).

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Description of an Instrument for Separation of Red Cells From Plasma and Measurement of Red Cell Volume

OSCAR DRIESSEN,[1] MARTIN S. HIGHLEY,[2] PETER G. HARPER,[2] ROBERT A. A. MAES,[3] and ERNST A. DE BRUIJN[4]

[1]University of Leiden, Leiden, The Netherlands, [2]Guy's Hospital, Department of Medical Oncology, London, UK, [3]University of Utrecht, NIDDR, Utrecht, The Netherlands, and [4]University of Antwerp, Laboratory of Cancer Research & Clinical Oncology, Universiteitsplein 1 (T-3), B-2610 Wilrijk, Belgium

Introduction

In order to determine the amount of a compound associated with red cells, the usual approach is to measure its concentration in whole blood and subtract from this the amount present in plasma, corrected for the hematocrit. However, when the compound is found in high concentrations in plasma relative to red blood cells, this approach can be very inaccurate and may even result in negative values when the erythrocyte associated concentration is calculated.

Obviously, if the plasma volume between red cells can be diminished, a more direct analysis of compounds associated with red cells can be performed. A sediment of red cells obtained at 10,000 × *g* will contain no more than 2% (v/v) of trapped plasma. Therefore, it should be possible to separate off and measure such a cell sediment, containing a constant volume of trapped plasma, with the concentration of compounds on the red cells unchanged by the procedure. Thereafter, compounds associated (1–4) with a known mass of red cells can be quantified. We describe here an instrument that we have designed to separate red cells from plasma and allow for the measurement of red cell volume.

Materials and methods

Reservoir A is inserted into container B, as shown in Figure 1. The reservoir is fitted tightly into the container so that the small orifice of the reservoir (Figure 1, #1) is sealed by the o-ring in the container (Figure 1, panel A, #2). The reservoir is filled with an aliquot of blood and the unit placed in a swing out rotor. By centrifuging at high speed (>5000 × *g*) red cells are forced into the narrow part of the reservoir (Figure 1, #3), into the closed small channel (Figure 1, #4) and into the bottom of the broad part of the reservoir (Figure 1, #5). The amount of trapped plasma remaining between the cells may be essential to maintain the same equilibrium between cell and plasma concentrations as exists in whole blood. After the first centrifugation some supernatant plasma is removed for independent analysis. Before the second centrifugation, reservoir A is detached by turning A in B, so that the o-ring (Figure 1, #2) no longer occludes the orifice (Figure 1, panel B, #1). During the second centrifugation, the flow resistant red cell sludge, together with the remaining supernatant plasma, is forced out of reservoir A into the bottom of container B, leaving behind a defined volume of almost pure red cells in the narrow part of A (Figure 1, #3). This mass of cells is transferred to a centrifuge tube by centrifuging the inverted reservoir alone.

Proteins often bind red cell associated compounds more avidly than red cells themselves. In these instances compounds can be *quantitatively* extracted by a blank albumin solution from the cell sediment (5).

To test the accuracy of the instrument, the closed reservoir (Figure 1, panel A) was filled with 0.5 mL of fresh anticoagulated blood collected in lithium-heparin containing vacutainers and centrifuged in a swing out rotor at a speed of 5000 × *g*. The distance from the bottom of the buckets to the central centrifuge axis was 15 cm. Immediately after the first run a second one was performed with the instrument as depicted in Figure 1, panel B.

The mass of cells collected in the narrow part of the reservoir (Figure 1, #3) was then transferred into a preweighed tapered empty centrifuge tube by centrifuging the inverted reservoir alone. By reweighing the centrifuge tube afterward, the mass of the cell sediment was obtained. In this way six instruments chosen at random were tested.

Results and discussion

The collected mass of cells was 109.5 mg (±0.2 mg, $n = 6$), indicating that the method is reproducible.

Correspondence: Dr. E.A. de Bruijn.
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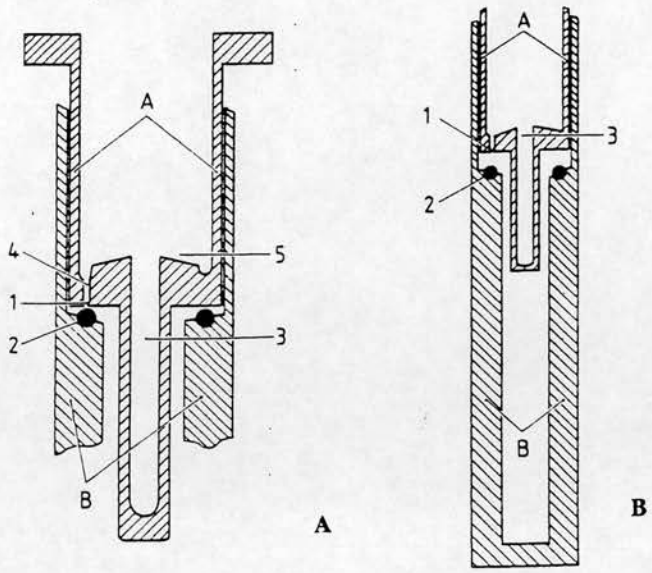

Figure 1 — The MESED (measuring sediment) instrument. Panel A, Closed position; panel B, open position.

This weight depends on the exact dimensions of the narrow part of the reservoir, on the centrifugal force applied, and on the density of the cells, the density of normal erythrocytes being 1.0964. It is important that *fresh anticoagulated* blood collected in vacutainers be used. The presence of microclots or cold precipitates predisposes to blockage of the narrow orifice (Figure 1, #1; ID 0.45 mm), thereby preventing the removal of surplus material from the reservoir during the second centrifugation.

The plasma concentration of a compound will often be higher than that associated with cells in the blood, and consequently a correction must be made for the plasma trapped in the sediment. Therefore the amount of trapped plasma should be determined once under standardized conditions with an extracellular marker. Using ^{51}Cr-EDTA the volume of trapped plasma appeared to be 3.4–3.9% (v/v) (n = 5) in a range of 5000 × g/5 min–5000 × g/3 min. The collection of supernatant plasma after the first centrifuge run and its subsequent analysis is required for this correction.

The technique we have described has practical applications. In previous studies (5,7,8) it was shown that for a given compound the elimination half-lives from the various components of whole blood are quite different. Elimination from the erythrocyte is as rapid or faster than that from the plasma water, in contrast to the lower elimination from plasma proteins. For example, approximately 2–24 h after the intravenous administration of phenytoin, the half-life of the protein bound fraction is 67.9 h, that of the unbound fraction 38.5 h, and that of the red cell fraction 21.4 h, these values being significantly different (8). Although the amount bound to erythrocytes is less than that bound to plasma proteins, the faster elimination from erythrocytes, coupled with the high number of these cells in the circulation (45% v/v) implies that erythrocytes are important carriers of such compounds. In the case of phenytoin, calculations showed that between 1 and 5 h after administration, approximately 50% of the drug leaving the blood originated from erythrocytes.

The amount of trapped plasma determined with ^{51}Cr-EDTA increased from 3.4–3.9% at 5000 × g to 5–8% at 1000 × g. The data stress the importance of calibration and proper choice of centrifugation conditions. The amount of trapped plasma (9–11) appeared to be somewhat higher than that reported by Siebers *et al.* (11). This might be due to differences between the behavior of ^{51}Cr-EDTA (9,10) and (Co^{2+})-EDTA (11).

In conclusion the instrument presently described could have applications in the following fields:

1. The study of the relationship between the degree of red cell binding of a compound and the observed clinical effects of that compound.
2. The study of the mutual displacement of body constituents, xenobiotics, and foodstuffs in fractions of whole blood.
3. The determination of the distribution of a compound and its metabolites between red cells and plasma, for example, antimalarial drugs and vitamins; the study of red cells as bioreactors.

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Short Communication

Formation of Chloroethylamine and 1,3-Oxazolidine-2-one following Ifosfamide Administration in Humans

Ifosfamide is an oxazaphosphorine alkylating agent used extensively in the treatment of malignant disease. It is a prodrug requiring activation in the liver by the cytochrome P450 system. Two routes of metabolism are possible; first, activation to form 4-hydroxyifosfamide, with the subsequent formation of IPM[1]; and second, dechloroethylation to produce 2-DCEI, 3-DCEI, and chloroacetaldehyde. Carboxyifosfamide, ketoifosfamide, and 4-thioifosfamide are also generated (fig. 1). The structural isomer cyclophosphamide follows a similar metabolic fate, but NNM has also been found after intravenous administration in humans. In one report, the source of NNM was not clear, because cyclophosphamide, 4-ketocyclophosphamide, carboxyphosphamide, and phosphoramide mustard were all capable of decomposing to NNM during the freezing and thawing of plasma (1). When phosphoramide mustard is injected into mice NNM is formed, and this in turn yields 3-(2-chloroethyl)-1,3-oxazolidine-2-one (2). In the case of IPM, an equivalent transformation would lead to chloroethylamine and 1,3-oxazolidine-2-one; chloroethylamine has alkylating activity, but only one-fifth the potency of NNM (3). We describe the detection of chloroethylamine and 1,3-oxazolidine-2-one in plasma following oral and intravenous administrations of ifosfamide in humans.

Four patients were studied. Patients 1 and 3 received 3 g m^{-2} ifosfamide (5.35 g and 4.7 g, respectively) and 2 g m^{-2} mesna (sodium-2-mercapto-ethane sulfonate) as a 6-hr intravenous infusion in 500 ml 0.9% sodium chloride; intravenous boluses of 800 mg mesna were given immediately before and 8 hr after the infusion. Patients 2 and 4 were given ifosfamide 500 mg orally as a gelatin capsule with a 300 mg film-coated mesna tablet. None had received prior treatment with oxazaphosphorines. Blood samples were taken at intervals during and following the intravenous infusion for ~24 hr after completion and for ~6 hr after oral administration. Plasma was separated immediately by centrifugation and stored at −20°C until analysis.

Ifosfamide and its metabolites were determined using GC/MS as previously described (4). Isolation of ifosfamide, IPM, 2-DCEI, 3-DCEI, carboxyifosfamide, and ketoifosfamide was performed by solid-phase C_{18} extraction at pH 4. Two derivatization steps were used: methylation of the acidic groups of carboxyifosfamide and IPM, followed by trifluoroacetylation of unhindered phosphoramide groups. Analyses were then conducted using negative chemical ionization by electron capture. Chloroethylamine and 1,3-oxazolidine-2-one were isolated by liquid extraction with ethyl acetate at pH 10. One-step derivatization using trifluoroacetic anhydride was used and GC/MS performed in the electron impact ionization mode. The structures of the trifluoroacetyl derivatives of chloroethylamine and 1,3-oxazolidine-2-one with their mass spectra are given in fig. 2.

The plasma metabolite profiles of patients 1 and 2 are shown in fig. 3. Peak levels of 2-DCEI, 3-DCEI, and carboxyifosfamide occurred ~6 hr after the end of the intravenous infusion, and only a small amount of ketoifosfamide was seen. Peak concentrations of IPM, chloroethylamine, and 1,3-oxazolidine-2-one coincided with the end

[1]Abbreviations used are: IPM, isophosphoramide mustard; 2-DCEI, 2-dechloroethylifosfamide; 3-DCEI, 3-dechloroethylifosfamide; NNM, nornitrogen mustard.

Send reprint requests to: Dr. M. S. Highley, Department of Medical Oncology, Guy's Hospital, St. Thomas Street, London SE1 9RT, UK.

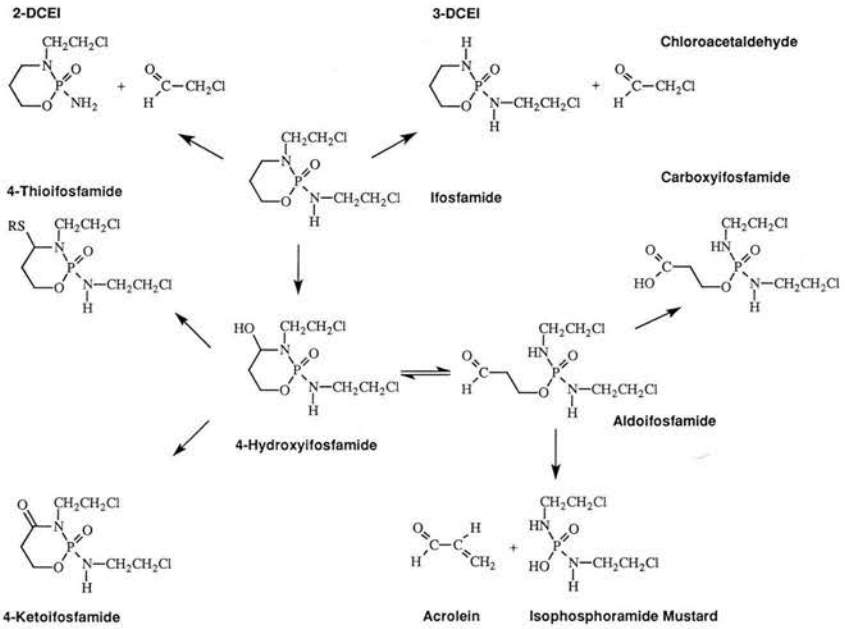

FIG. 1. *Metabolism of ifosfamide.*

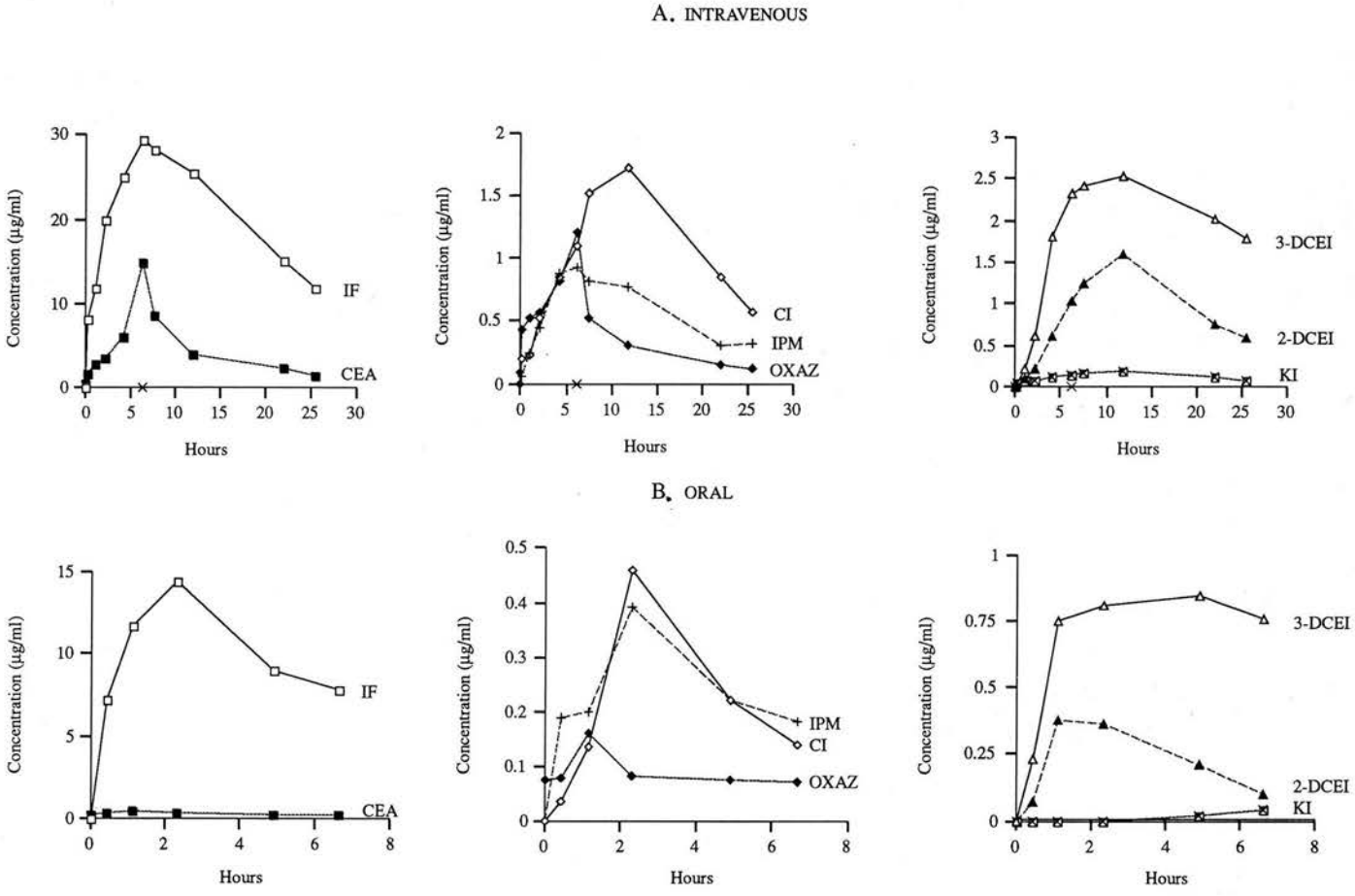

FIG. 3. *Plasma concentration-time curves following ifosfamide administration.*

(*A*) Intravenous (patient 1); (*B*) oral (patient 2); IF, ifosfamide; CEA, chloroethylamine; KI, ketoifosfamide; CI, carboxyifosfamide; OXAZ, 1,3-oxazolidine-2-one; ×, marks the end of the infusion.

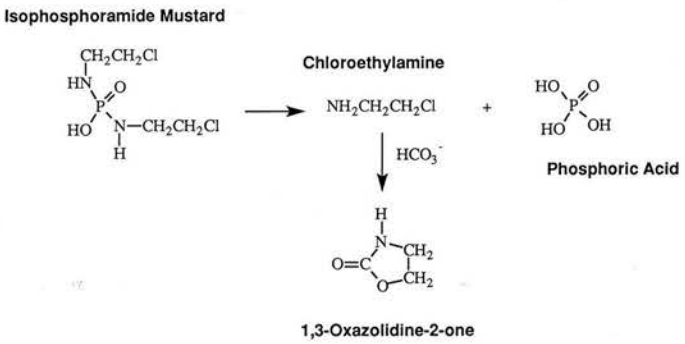

FIG. 4. *Conversion of IPM to chloroethylamine and 1,3-oxazolidine-2-one.*

of chloroethylamine were found compared with IPM, suggesting the presence of an additional route of chloroethylamine formation. A similar difference was observed between patients 3 and 4; after intravenous infusion, the peak concentration of chloroethylamine was 9.73 μg/ml and ifosfamide was 20.37 μg/ml, compared with respective values of 0.43 and 10.45 μg/ml following oral administration.

Significant quantities of NNM have been reported following the intravenous administration of cyclophosphamide, and, as an explanation, the further metabolism of carboxyphosphamide was postulated (1). However, the plasma profiles obtained after ifosfamide administration do not indicate carboxyifosfamide as a source of chloroethylamine. The relatively high levels of chloroethylamine generated after intravenous infusion compared with oral dosing, with a curve following the ifosfamide plasma profile, infer that significant hydrolysis of ifosfamide itself is occurring, either before or after direct injection into the circulation. In aqueous solution at 70°C, cyclophosphamide hydrolyzes to NNM, *N*-propanolamine, and phosphoric acid (8). Other hydrolytic products have been described following more prolonged exposure of cyclophosphamide solutions to a temperature of 37.5°C, including 2-hydroxy-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide (9). We therefore explored the possibility that ifosfamide could behave in a similar fashion.

Aqueous solutions of ifosfamide (1000 μg/ml and 100 μg/ml, respectively) were buffered at pH 4, 7 and 10; maintained at 40°C for 5 hr; and an aliquot extracted with 6 ml of dichloromethane. The solvent was evaporated and the dry residue treated with diazomethane in ether, the solvent again evaporated, and the residue dissolved in 500 μl of methanol. The methanol solution was then injected into the GC/MS. In all six samples, three peaks were detected: using electron impact ionization, the first was identified as phosphoric acid methyl ester; the second as 3-(2-chloroethyl)-2-methoxy-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide (**I**); and the third as ifosfamide. Chloroethylamine was also detected in all six solutions, as previously described. These findings suggest that hydrolysis of ifosfamide to chloroethylamine and 3-(2-chloroethyl)-2-hydroxy-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide (**II**) is taking place. In a further experiment, an aqueous solution of ifosfamide (2000 μg/ml) was main-

peak chloroethylamine/ifosfamide ratios of 50.2% and 50.1% (w/w) after intravenous administration indicate that chemical hydrolysis is significant in the plasma at body temperature. Corresponding values after oral dosing are 4.9% and 8.2% (w/w). The higher levels of chloroethylamine found in the plasma after intravenous ifosfamide probably reflect the nature of the hydrolysis reaction, the rate of which is dependent on the concentration of ifosfamide and therefore the administered dose. Differences in the age or stability of ifosfamide powder within gelatin capsules or glass vials may also be contributory, but an analysis of the capsules was not performed.

In conclusion, chloroethylamine is an important product of ifosfamide degradation and seems to be formed predominantly by hydrolysis. Significant quantities are detected *in vivo* following high-dose ifosfamide administration. Further work is continuing on the presence of chloroethylamine in ifosfamide powder and the determination of compounds **II** and **V** in plasma.

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Departments of Medical Oncology, Guy's (M.S.H., P.G.H.) and St. George's (J.M.) Hospitals; M. S. Highley, G. Momerency, K. Van Cauwenberghe
Department of Radiotherapy (P.B.), Royal Marsden Hospital; A. T. Van Oosterom, E. A. de Bruijn
Laboratory for Cancer Research and Clinical Oncology (G.M., K.V.C., A.T.V.O., E.A.d.B.), University of Antwerp; and R. A. A. Maes, P. Blake, J. Mansi
Netherlands Institute of Drug and Doping Research (R.A.A.M.), University of Utrecht P. G. Harper

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Erythrocytes and the Transport of Drugs and Endogenous Compounds

Martin S. Highley and Ernst A. De Bruijn

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Review

Erythrocytes and the Transport of Drugs and Endogenous Compounds

Martin S. Highley[1] and Ernst A. De Bruijn[2,3]

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This review considers the significance and measurement of endogenous compounds and drugs on erythrocytes. Part I examines literature examples where a *direct* measurement of hydrocortisone, phenytoin and valproate was performed on unwashed red cells in vitro and in vivo, showing a consistent contribution of the erythrocyte fraction to the transport of these compounds. *In vitro* partition experiments using systems composed of plasma water, plasma proteins and erythrocytes are discussed. When spiked blood is diluted with blank autologous plasma water, erythrocytes always discharge the compound over-proportionally compared to plasma proteins. *In vivo,* during the distribution phase, the elimination half-life from the erythrocyte is the same as or shorter than that from plasma water, and substantial amounts of drug leaving the circulation originate from erythrocytes. In Part II, the transfer of compounds is considered and evidence for the facilitated exchange of red cell associated substances between the erythrocyte and capillary endothelium presented. Situations where a failure to analyse the erythrocyte compartment leads to the loss of vital information are identified. Part III explores methods for analysing erythrocyte associated substances, most commonly indirect *calculation,* or analysis of *washed* erythrocytes. A *direct* determination is rarely performed, but one such method, allowing concurrent plasma analysis, is discussed. An instrument collects a *fixed and known* quantity of a maximally compressed cell mass, without disturbing the equilibrium between cells and plasma. To isolate compounds associated with the mass of erythrocytes, the red cell sediment can often be extracted quantitatively into a blank protein solution.

KEY WORDS: biological transport; erythrocytes; plasma; protein binding; blood specimen collection; valproic acid; phenytoin; hydrocortisone.

INTRODUCTION

Quantitatively, the three most important transport fractions of blood are plasma water, plasma proteins and cells, mainly erythrocytes. In the laboratory, this three compartmental system, equilibrating in the circulation in the dark and at 37°C, is reduced for practical purposes to the two compartmental system of plasma and analysed at room temperature as if it had only one compartment. The information present in the three separate fractions of whole blood is therefore combined into one value. In the case of a compound which is bound to plasma proteins, this composite value is misleading as a change in the free fraction present in plasma water can be hidden by the large quantity present on proteins. Binding to erythrocytes also occurs, but even less consideration has been given to the role of these cells in the transport of compounds other than oxygen.

There are three reasons for this neglect of the erythrocyte and subsequent lack of knowledge. Firstly, conceptual difficulties exist, as illustrated by the following statement: "The erythrocyte has a cell wall, so it really has its own compartment. Distribution in body fluids does not take effect versus the fraction free in whole blood, but versus the free concentration in plasma water. From the pharmacokinetic point of view, I would say that the red blood cells have the lowest priority" as discussed by a pioneer on erythrocyte studies, M. Ehrnebo (1). As a result, the transport role of erythrocytes is almost invariably investigated using *washed* erythrocytes, as if these cells were indeed separate compartments, and regardless of the fact that washed erythrocytes do not exist in the circulation. Secondly, the transport function of red cells is influenced by physiological factors as the role of erythrocytes will often become more apparent when protein binding is saturated, and thirdly, bioanalytical limitations restrict the investigation of the transport role of erythrocytes.

In the first part of the review, three reports from the literature on the drugs valproate and phenytoin, and the endogenous compound hydrocortisone, are described in detail and the data re-evaluated. All three sudies illustrate that when the above considerations are addressed, erythrocytes do in fact show a consistent and important transport function.

In part II, a review of the mechanisms and significance of substance transport by red cells, and subsequent exchange at the capillary endothelium, is presented.

Part III discusses approaches to the analysis of red cell associated substances, including a method for the analysis of

[1] Department of Medical Oncology, Guy's Hospital, London, UK.
[2] Laboratory for Cancer Research and Clinical Oncology, University of Antwerp, Universiteitsplein 1 (T-3), B-2610 Wilrijk, Belgium.
[3] To whom correspondence should be addressed.

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drugs or endogenous compounds both directly on red cells and in the plasma of a single blood sample.

PART I

THE TRANSPORT OF SUBSTANCES WITHIN THE MAIN BLOOD FRACTIONS AND THE CONTRIBUTION OF THE ERYTHROCYTE (Three examples from the literature)

Methods of Erythrocyte Investigation

The use of appropriate methods is of vital importance for demonstrating the transport role of erythrocytes. The essence is to measure the compounds present in or on unwashed erythrocytes directly. Three studies in which such an approach was used (2–5) are reviewed in detail. They concern the compounds valproate, an antiepileptic drug and small fatty acid; phenytoin, also an antiepileptic drug; and the steroid hormone hydrocortisone. Both in vitro and in vivo work was described.

The method used to process the erythrocytes in these studies involved harvesting blood into haematocrit capillaries and separating erythrocyte sediments by centrifugation at 10,000 g. The capillaries were then cut with a glass knife, and both the length of the cut fragment and its weight determined. Knowing the weight per unit length of capillary tubing, the mass of the cells was obtained. The cell mass was extracted and its drug content analysed. From these measurements a known concentration in w./v. of cell mass was derived. A correction for the difference in density between red cells and water was not made. During sample pretreatment, care was taken to maintain the same equilibria as present in whole blood or an erythrocyte suspension in plasma water. Hence after sampling, the haematocrit capillaries were centrifuged immediately and the sediment separated from plasma or plasma water. The concentration of drug in the red cell sediment was then adjusted for the residual plasma trapped between the cells. This was determined using ^{14}C-inulin as an extracellular marker and appeared to be 2.0%, in accordance with the literature (6). During sample pretreatment this trapped plasma may be important to maintain the equilibrium that exists in the circulation. The quantity of drug bound to plasma proteins was calculated by subtracting the quantity in plasma water from that in plasma. In vitro, it was confirmed that the total drug recovery from all the fractions was equal to the amount added to blood, plasma or the mixture of red cells and plasma water.

In vitro Partition of Phenytoin, Valproate and Hydrocortisone Between Plasma Water, Proteins and Red Cells

Tables I to III, constructed from the original data of Driessen et al (2–5), show the distribution of these compounds between the three main blood fractions at 20°C; concentrations are expressed in weight per volume of blood, plasma, plasma protein, plasma water or erythrocytes.

(a) Distribution within the Binary System of Plasma Water and Plasma Proteins, i.e. Plasma

Table I shows the plasma concentrations of phenytoin, valproate and hydrocortisone, and the concentrations bound to protein compared to those in plasma water, expressed as the plasma protein/plasma water concentration ratio. As expected, the higher the concentration in spiked plasma, the lower the plasma protein/plasma water ratio of the compound, indicating that the plasma proteins are saturable with all three compounds.

(b) Distribution within an Erythrocyte and Plasma Water Binary System

This is an artificial mixture, in which proteins adhering to erythrocytes are stripped from these cells by washing with autologous plasma water, *before the compounds are added.* Following distribution of the compound between the two fractions, the erythrocytes are not subjected to further washing. The observed red cell/plasma water concentration ratios, over a similar concentration range to that described in Table I, are shown in Table II.

In contrast to the data shown in Table I, the red cell/plasma water ratios are constant, indicating that erythrocytes cannot be saturated by these compounds, not even in the high and toxic concentration ranges used. Compounds which are distributed only in the water phase, and to the same extent both inside and outside the erythrocyte, should have a red cell to plasma water concentration ratio of approximately 0.65; substantially higher ratios indicate accumulation within or on the surface of the red cell.

(c) Distribution within a Ternary System of Plasma Water, Plasma Proteins and Erythrocytes, ie Whole Blood

Table III presents the effect of increasing concentration on the distribution ratios within whole blood.

As the concentration in spiked blood increases, the concentration in plasma water increases relatively to that on plasma protein. Comparing the data in Table III with that in Table I, it must be appreciated that the plasma fraction is now approximately 55% of the total volume, and that the erythrocytes occupy the remaining 45%. Consequently, in addition to competition between the binding sites of the different fractions, the relative volume of these fractions will also influence the observed concentration ratio. This hinders a direct comparison of the observed plasma protein/plasma water ratio in plasma (Table I), with that in blood (Table III). It is striking that as the total concentration in the system increases, the erythrocyte/plasma water ratio remains constant for each drug in the binary system (Table II), and is also constant for phenytoin and hydrocortisone in the ternary system (Table III). *This indicates that for these two compounds, the concentrations on the erythrocytes and in plasma water increase proportionally.* As varying concentrations of phenytoin and hydrocortisone do not alter the red cell/plasma water ratio, the erythrocyte concentration is proportional to the concentration in plasma water.

In Table III, the erythrocyte/plasma water concentration ratios for hydrocortisone and phenytoin appear slightly increased and decreased respectively, in comparison with those in Table II. One must be cautious interpreting this difference, as it seems that the same blood sample was not used in the two experiments, and it is known that other substances, e.g. non-esterified fatty acids, can change the equilibrium between compartments (7–11). Moreover, in the ternary system, the equilibrium between red cells and plasma water was established

Table II. Erythrocyte and Plasma Water Binary System (Hematocrit 0.39 – 0.50; Temperature 20°C)
System concentration and erythrocyte/plasma water concentration ratio (sd)

Valproate		Phenytoin		Hydrocortisone	
System concentration (μg/ml)	red cell/plasma water ratio n = 3	System concentration (μg/ml)	red cell/plasma water ratio n = 3	System concentration (μg/ml)	red cell/plasma water ratio n = 3
2.7	0.68 (0.03)	0.8	4.5 (0.2)	0.18	2.1 (< 0.1)
24.7	0.68 (0.01)	5.8	4.5 (0.1)	0.68	2.0 (0.1)
44	0.60 (0.02)	10.8	4.6 (0.2)	1.18	2.0 (0.1)
66	0.70 (0.01)	20.8	4.5 (0.1)	5.18	2.1 (0.1)
88	0.68 (0.02)	50.8	4.5 (< 0.1)	10.18	2.1 (0.1)
132	0.70 (0.01)	100.8	4.5 (0.2)		
176	0.67 (0.01)				
220	0.69 (0.02)				
978	0.70 (0.01)				

room temperature between 3 and 10 minutes after pooling (3). There are other observations in the literature supporting the slower equilibration of phenytoin. Graves et al reported a consistently lower concentration of phenytoin in capillary serum than in simultaneously sampled venous serum. This difference did not exist for phenobarbital, and the source of this discrepancy was not found (17). In another study, an escalating dosage regimen of phenytoin resulted in significantly lower plasma concentrations compared with the same dose given in a reducing regimen (18). This hysteresis loop may be explained by a slow equilibration of phenytoin between tissue cells and plasma. A prolonged time to equilibrium of 60 minutes, between blood cells and plasma, has also been described for pirarubicin (19).

However, for the majority of drugs in large vessels at 37°C, and in clinical samples drawn from them, it seems likely that red cells are in equilibrium with plasma.

Table III. Plasma Protein, Plasma Water and Erythrocyte Ternary System (whole blood) (Hematocrit 0.39 – 0.5; Temperature 20°C)
Blood concentration with plasma protein/plasma water and erythrocyte/plasma water concentration ratios

Blood Concentration (μg/ml)	Plasma protein/plasma water ratio n = 3	Erythrocyte/plasma water ratio n = 3
	Valproate	
2.7	17.0 (0.6)	0.33 (0.01)
23.1	6.3 (1.1)	0.35 (0.04)
44	7.7 (0.4)	0.24 (0.02)
66	6.0 (0.3)	0.37 (0.02)
88	4.4 (0.3)	0.40 (0.04)
132	2.9 (0.3)	0.51 (0.05)
220	1.5 (0.2)	0.55 (0.03)
978	0.3 (< 0.1)	0.66 (0.04)
	Phenytoin	
0.8	12.7 (0.5)	3.7 (0.3)
5.8	12.8 (0.2)	4.0 (0.3)
10.8	12.0 (0.4)	3.9 (0.3)
20.8	10.8 (0.1)	3.8 (0.3)
50.8	8.6 (0.3)	3.9 (0.3)
100.8	6.4 (0.1)	4.1 (0.2)
	Hydrocortisone	
0.18	7.2 (0.3)	2.3 (0.1)
0.68	3.2 (< 0.1)	2.4 (0.1)
1.18	2.7 (0.1)	2.4 (0.1)
5.18	2.1 (< 0.1)	2.4 (0.1)
10.18	2.1 (0.1)	2.4 (0.1)

In vivo Partition of Phenytoin, Valproate and Hydrocortisone Between Plasma Water, Proteins and Red Cells

Figures 1, 2 and Table V, also compiled from Driessen et al (2,3,5), illustrate the in vivo distribution of phenytoin, valproate and hydrocortisone.

Figure 1 shows the log concentration time curves of valproate in the three blood fractions, after an oral dose of 2.4 g

Table IV. Distribution of Substances Between Blood Compartments in vitro, before and After Expansion of Blood with Blank Autologous Ultrafiltrate

	Before	After	Change
Valproate (VPA)			
volume of whole blood	2.0 ml	2.9 ml	+45%
total amount of VPA	151.0μg	151.0μg	0%
VPA in plasma water	16.8%	29.4%	+75%
VPA on proteins	72.2%	63.4%	−12.7%
VPA on erythrocytes	7.7%	4.7%	−40%
Phenytoin (DPH)			
volume of whole blood	2.0 ml	2.9 ml	+45%
total amount of DPH	21 μg	21 μg	0%
DPH in plasma water	5.4%	8.9%	+65%
DPH on proteins	76.1%	76.6%	+0.7%
DPH on erythrocytes	17.6%	16.5%	−6%
Hydrocortisone (HCOR)			
volume of whole blood	1.5 ml	2.4 ml	+60%
total amount of HCOR	97.5 ng	97.5 ng	0%
HCOR in plasma water	11.1%	18.6%	+68%
HCOR on proteins	68.0%	64.7%	−5%
HCOR on erythrocytes	20.2%	16.3%	−19%

necessarily imply that compounds are unavailable to the tissues, but with the exception of oxygen the transport mechanisms have been poorly defined. For most compounds showing an association with erythrocytes, it is not known whether they primarily bind to the outside of the erythrocyte, penetrate the cell membrane, or if both mechanisms are operative. Despite these uncertainties, erythrocytes may be of great importance in transferring such compounds to the tissues. This section discusses the erythrocyte in the context of the classic equilibrium model of plasma proteins, plasma water and tissues.

The Starling Hypothesis

In 1895, Starling introduced the concept of the colloid osmotic pressure in his article: "On the absorption of fluids from the connective tissue spaces" (22). At the same time Hamburger called the absorption of fluid from the tissue spaces "molecular imbibition" (22). The absorbed fluids also contained dissolved electrolytes. The movement of tissue water containing dissolved compounds into a capillary through its endothelium, as a result of the colloid osmotic pressure, was also visualised as occurring in the reverse direction *towards* tissues by virtue of the arterial pressure. Nevertheless, it is not certain if compounds need to be dissolved in plasma water to pass a capillary endothelium into the tissues. An equilibrium between blood and tissues based solely on compounds dissolved in the plasma water phase is a commonly held view in bioscience, (e.g. see the quote of Ehrnebo in the introduction), and is often used in kinetic models (9,23). However, compounds bound to transport proteins in blood may use an additional pathway. Pardridge et al suggest that there are circumstances where the influx of a compound from capillaries into tissues is greater than can be accounted for by the influx from plasma water alone. Their model assumes that an inhibition of protein binding occurs on the capillary endothelium, thereby releasing the compound, and as a result tissue levels correspond more closely to the protein bound fraction than to the free fraction (24,25).

This concept is just as important when considering erythrocytes, as tissues on the other side of a capillary endothelium, or the capillary endothelium itself, may be more exposed to compounds accumulated on these cells (26,27). In the studies reviewed in part I, it was calculated that approximately 40% of the valproate leaving the circulation in the distribution phase originated from erythrocytes; for phenytoin, between one and five hours after administration, this figure was approximately 50%; and for hydrocortisone the value was 28%, between one and twenty minutes after injection. This is in spite of the much lower concentration of drug in the red cell fraction compared to the plasma protein fraction.

Plasma Water as the Central Compartment

Figure 3 shows a dynamic model of whole blood with tissues. It is assumed that the tissues are "empty", and that the redistribution of substances from tissue to blood is negligible.

In this model the plasma water is the central compartment. In a situation of excessive outflow to the tissues, as illustrated in the figure, the concentration in the plasma water compartment depends on the influx from proteins and cells. For a substance bound to plasma protein, the plasma water half-life will be the same as that on proteins when the red cells are empty, whereas it equals that on red cells when loaded erythrocytes discharge and proteins do not deliver substance in any appreciable amounts to plasma water. As binding to proteins is often stronger than to erythrocytes, the half-life measured in plasma water in the case of "empty" tissues will often lie between the longer one of plasma proteins and the shorter one of red cells (see part I, Table V), provided both of these compartments are delivering the substance to plasma water. It seems that the mass of a substance transferred from plasma proteins or red cells to tissues, as depicted in Figure 3, depends firstly, on the relative binding force exerted by these fractions, and secondly, on their storage capacity. A maximum capacity for erythrocytes is sometimes recorded in the literature (28), but often such a maximum cannot be determined (2–4,29,30). Both saturable (hyperbolic) and non-saturable (linear) uptake processes have been described (20,23,31), and the non-linear release from erythrocytes of thiazide diuretics, such as chlorothiazide, on account of binding to intra-erythrocytic carbonic anhydrase, has been well documented (21).

It is likely that the hypothetical transport of substances directly from the red cells to tissues, (dashed arrow in Figure 3), will be difficult to demonstrate using blood samples from large blood vessels.

The Role of the Erythrocyte

As well as generating the colloid osmotic pressure, the higher protein content of plasma, compared to the content of interstitial water, may form a "chemical barrier", to some extent isolating tissues from many compounds in the circulation, and hence from the exterior and each other. Only when the capacity of the plasma proteins is exceeded, are cells—initially blood cells—confronted by higher concentrations. The ability of the erythrocyte to take up and transport molecules is not compromised by its cell wall, provided that such molecules, located inside or outside the wall, can be exchanged with the plasma water fraction or the endothelium during passage through the capillary, a process well illustrated by the transport of oxygen.

Hence the concentration on erythrocytes will often not be significant at low plasma concentrations, and it is only as the plasma concentration increases that erythrocytes carry a physiologically or clinically relevant load. This occurs when plasma protein binding is saturated, especially locally following an intravenous bolus injection, or in blood flowing through the microvilli and transporting substances from the gut lumen to the portal vein. The experimental conditions described in part I, where blood is diluted with autologous plasma water, simulate a capillary with a discontinuous endothelium, found for example in the liver. Erythrocytes, loaded with compounds absorbed from the intestinal microvilli, pass through the portal vein and enter the liver sinusoids, which are lined with fenestrated endothelial cells; when they come into direct contact with the intercellular fluid they release their cargo more readily than the plasma proteins (32) (see Table IV, Part I).

Physical factors, including temperature, affect equilibration within whole blood, but to our knowledge there are only two studies in the literature where partition was observed methodologically at 37°C (19,23). In vitro, the blood to plasma concentration ratio of pirarubicin is 1.8 times higher at 37°C than at 25°C, after 60 minutes incubation, suggesting increased uptake by erythrocytes (19). Furthermore, the binding of phe-

darone, a metabolite of amiodarone (48), or cyclophosphamide (49). A physiological model of hydrocortisone transport has been described, in which the erythrocyte is central to the delivery of this compound to tissues, on account of the faster rate of dissociation of erythrocyte bound hydrocortisone compared to that bound to cortisol binding globulin (CBG) (14). One would also expect that in certain abnormal conditions (e.g. hyperbilirubinaemia, uremia), when large quantities of pathophysiological products bind to plasma proteins, the secondary transport system will be loaded more heavily (10,13).

Therefore, in the case of certain compounds and in some pathophysiological conditions, it is worthwhile investigating the relationship between the concentration on plasma proteins, the steady state load on erythrocytes and the physiological consequences; some desired or undesired effects may be more closely related to erythrocyte load than to the concentration on (nearly saturated) plasma proteins. For example, in neonates with erythroblastosis foetalis, an unconjugated plasma bilirubin concentration of 300 μmol/l or more leads to the uptake of bilirubin into the central nervous system, which may result in kernicterus. This pivotal plasma concentration may also be accompanied by a manifest loading of erythrocytes.

PART III

THE ANALYSIS OF THE ERYTHROCYTE COMPARTMENT

Methods Used in the Literature

After collecting a blood sample it is easy to obtain plasma by centrifugation, but the separation of plasma water from plasma is more laborious and not routinely performed in clinical laboratories. The degree of plasma protein binding of a substance is calculated by subtracting the amount present in the plasma water, called the free fraction, from that present in total plasma.

The red cell fraction presents the most obstacles when analysing the three main blood fractions, as the residual cell sediment formed following the centrifugation of blood is difficult to analyse quantitatively using normal volumetric procedures. One approach to this problem is to measure the volume of the cell sediment in a calibrated centrifuge tube. However it is then necessary to wash the sediment with saline or buffer several times before analysis, in order to remove the plasma trapped between the cells; this has the disadvantage of disturbing the normal equilibrium between red cells and plasma water, as presented in Figure 3 Part II. The effects of such a washing procedure on the results subsequently obtained are rarely mentioned, and to our knowledge the washing itself is never analysed. A second approach, in which the erythrocytes remain in their natural environment, but where the analysis is indirect, is also common in the literature. The amount associated with red cells is *calculated* by subtracting the amount present in plasma, corrected for the haematocrit, from that present in whole blood (not a medium in which concentrations are routinely measured). As the quantity associated with red cells is often small compared to the plasma concentration, two large numbers are used to determine a small one. This is an inaccurate method, and occasionally even negative values are obtained when the erythrocyte associated amount is calculated. When analysing the erythrocyte compartment these problems are frequently not discussed, and how the recorded erythrocyte concentrations are obtained is not clear. Of 56 reports in the literature describing the analysis of the erythrocyte compartment, 30 employed the washing technique, 11 used subtraction, and in 15 the method was not evident.

In the three studies reviewed in part I, a known weight of erythrocyte sediment was harvested from haematocrit capillaries, and a correction made for the trapped plasma (2% v./v.). Although surmounting the limitations described above, the method is so inconvenient that it is not acceptable for routine use. Only one other example was found in the literature where a similar procedure was followed (Maling et al 1989) (48). As discussed in part II, there is sometimes a need for a direct analysis of the erythrocyte compartment and a more appropriate method, which also allows the simultaneous analysis of plasma, is now available (50) (see below). This has recently been used to investigate the transport of cyclophosphamide and 4-hydroxycyclophosphamide in blood (49), and further work is being performed with other oxazaphosphorines, the taxanes, antimetabolites, mitomycins and suramin.

Direct Analysis of the Erythrocyte Compartment

A direct determination of red cell associated compounds must be performed on *unwashed* erythrocytes, maintained in their natural environment, without disruption of the normal equilibrium existing between red cells and plasma in whole blood. Washing procedures strip the red cells of their outer layer, which may be important in the transport and exchange process. Before a direct analysis, the red cells must be compressed, reducing the amount of plasma between the cells to a fixed volume, ideally as low as possible, approximately 2% (6). The red cells need to be collected in a reproducible manner, and the volume of the collected cell mass must be known.

An instrument (US patent 1993, no. 5256314) meeting these criteria is described in the literature (50), (Figure 4).

A reservoir A is inserted into a container B, as shown in Figure 4. The reservoir is fitted tightly into the container so

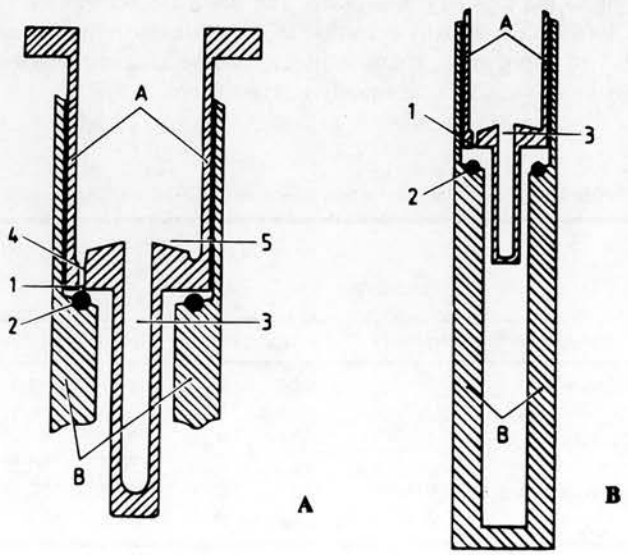

Fig. 4. The erythrocyte compartment.

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Original article

Activated oxazaphosphorines are transported predominantly by erythrocytes

M. S. Highley,[1] D. Schrijvers,[2] A. T. Van Oosterom,[3] P. G. Harper,[1] G. Momerency,[2] K. Van Cauwenberghe,[2] R. A. A. Maes,[4] E. A. De Bruijn[3] & M. B. Edelstein[5]

[1]*Department of Medical Oncology, Guy's Hospital, London, UK;* [2]*Laboratory for Cancer Research, Department of Medicine, University of Antwerp (UIA), Antwerp, Belgium,* [3]*Laboratory for Clinical Pharmacology and Bioanalysis, Department of Medicine, University of Leuven, Leuven, Belgium;* [4]*Department of Human Toxicology, University of Utrecht, the Netherlands;* [5]*Medical Service III, VAMC, Allen Park MI, USA*

Summary

Purpose: Oxazaphosphorines are metabolised by a variety of pathways, one of which leads to activation and the formation of alkylating compounds. However, the transport forms conveying activated oxazaphosphorines to the tumour cell have not been fully characterised. There is increasing recognition of the importance of the erythrocyte as a carrier of compounds in the circulation, and we have recently described higher concentrations of 4-hydroxycyclophosphamide within the erythrocyte compartment compared to plasma. We have now determined the concentrations of ifosfamide and seven of its metabolites in the plasma and erythrocytes of patients receiving a six-hour intravenous infusion of ifosfamide.

Patients and methods: Red cells from five patients, receiving a total of eight cycles of ifosfamide, were separated from plasma using the MESED instrument, and analysis of red cells and plasma performed using Gas Chromatography-Mass Spectrometry (GC/MS).

Results: The concentration of all compounds in the erythrocyte compartment was higher than or equal to those in plasma, and isophosphoramide mustard and carboxyifosfamide showed a particular affinity for the erythrocyte. The red cell fraction can contain as much as 77% of the total blood concentration of isophosphoramide mustard.

Conclusions: Erythrocyte associated isophosphoramide mustard is an important transport form of activated ifosfamide. Red cells may have a role in the delivery of activated oxazaphosphorines to tissues.

Key words: biological transport, blood specimen collection, erythrocytes, ifosfamide

Introduction

Cyclophosphamide and its structural isomer ifosfamide are oxazaphosphorine alkylating agents used widely in the treatment of many solid tumours. They are prodrugs requiring activation by 4-hydroxylation within the cytochrome P450 system, with the ultimate formation of both active and inactive metabolites. In the case of ifosfamide, isophosphoramide mustard is the final alkylating species, but other metabolites are thought to be responsible for significant toxic effects, such as haemorrhagic cystitis [1] and neurotoxicity [2].

The main site of formation of isophosphoramide mustard, and its predominant transport form within the circulation, is not certain. Two possibilities have been envisaged; firstly, the transport of 4-hydroxyifosfamide to tumour cells, which then convert this compound to isophosphoramide mustard, or secondly the formation of isophosphoramide mustard, primarily within the liver, but also within other tissues capable of activating ifosfamide, followed by transport within the circulation and direct penetration of tumour cells. The relative contribution of each of these mechanisms will influence the success of any attempt to administer isophosphoramide mustard itself as a chemotherapeutic agent, a potentially advantageous manoeuvre which should not lead to haemorrhagic cystitis or neurotoxicity.

Consequently, an improved knowledge of the processes of distribution of the oxazaphosphorines and their metabolites is needed. Blood consists of plasma and cells, and the vast majority of the cells are erythrocytes (approximately 45% of total blood volume). It is becoming clear that the erythrocyte is a significant carrier of substances in the circulation [3], but there is little data concerning the distribution of oxazaphosphorines and their metabolites throughout whole blood in humans.

New developments in the bioanalysis of the oxazaphosphorines have provided methods for the separation and accurate measurement of many metabolites in plasma [4, 5], and improved techniques of erythrocyte isolation have facilitated the study of the red cell compartment [6]. Recently, higher concentrations of 4-hydroxycyclophosphamide have been found within the erythrocyte compartment compared with plasma [7], and we have now investigated the distribution of ifosfamide and seven of its metabolites in the plasma and red cells of patients receiving an infusion of ifosfamide.

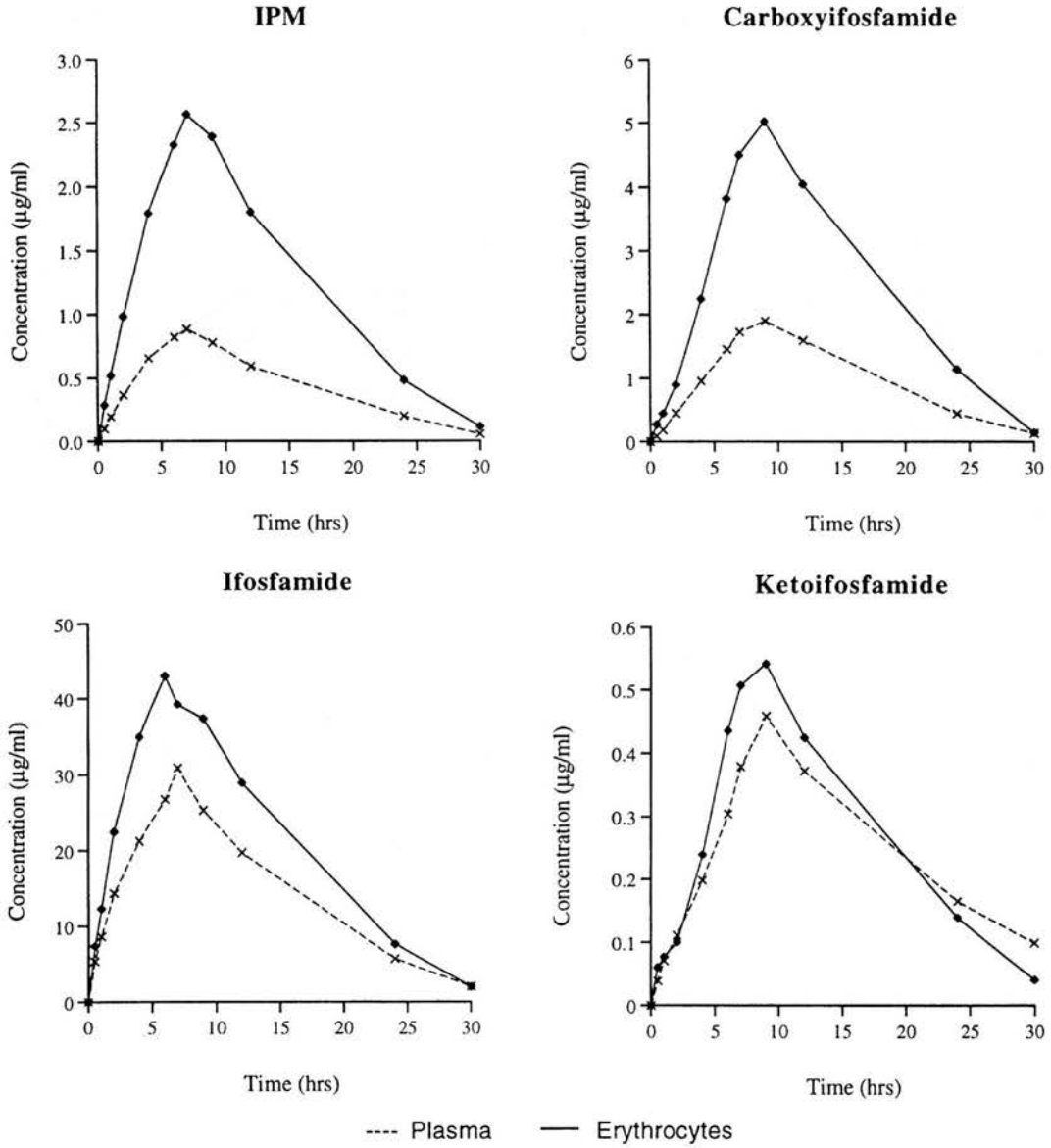

Figure 1. Mean concentration-time profiles for isophosphoramide mustard (IPM), carboxyifosfamide, ifosfamide and ketoifosfamide.

less, some groups have identified persistent, albeit small, amounts of 4-hydroxycyclophosphamide in the plasma of mice [8] and humans [9], proposing that low levels of 4-hydroxycyclophosphamide in plasma reflect intracellular trapping of this compound [8].

Other groups have argued that the mustard is the major transport form. Struck et al. [10] studied the activity of isophosphoramide mustard against murine tumours, found it comparable to ifosfamide, and postulated that isophosphoramide mustard acts by membrane alteration and/or non-DNA alkylation. The authors demonstrated that extracellularly delivered isophosphoramide mustard is an effective agent, with antitumour activity and selectivity *in vivo* comparable to ifosfamide and 4-hydroxyifosfamide.

Sladek has reviewed values of the ratio of phosphoramide mustard AUC to 4-hydroxycyclophosphamide AUC in the plasma of mice, rats and humans, following a dose of cyclophosphamide, and also the sensitivities of mice and human cell cultures to phosphoramide mustard and 4-hydroxycyclophosphamide [11]. He was able to calculate percentages of total cell kill hypothetically due to either circulating 4-hydroxycyclophosphamide or phosphoramide mustard, and found that the former compound was the circulating metabolite of major importance in the plasma, although the latter also made a contribution.

However, such studies have analysed plasma and have not considered the erythrocyte. We have investigated this compartment with a direct technique [6], avoiding the need for red cell washing or calculation of red cell concentrations from those in plasma and whole blood; our results show that ifosfamide and its metabolites enter the erythrocyte compartment freely, and that isophosphoramide mustard and carboxyifosfamide are concentrated most significantly within this fraction. At the time of the peak erythrocyte isophosphoramide mustard concentration in one patient, 82% of the total amount of

to compounds accumulated on these cells [13], and the compounds may enter tissues just as easily from erythrocytes as from plasma water. Flow through capillaries is intermittent, and red cells are momentarily held in intimate contact with the capillary endothelium, allowing direct exchange between erythrocytes and endothelial cells [14].

The structure and function of the tumour interstitium is significantly different from that of most normal tissues, and solid tumours can possess a special pattern of vascularisation [15]. For a solid neoplasm to survive, it must develop an adequate blood supply through the process of angiogenesis and then maintain the neovasculature so formed. In achieving this, endothelial cells in tumour vessels can proliferate more than twenty times faster than in normal vessels [16]. The action of cytotoxics on the capillary endothelium may contribute to their anti-tumour effect, as the disruption of a tumour capillary can lead to substantial tumour cell necrosis. Ifosfamide is used particularly in the treatment of solid tumours, and the intimate contact between the capillary endothelium and erythrocytes containing high concentrations of isophosphoramide mustard may be important. In a study of ten patients treated with cyclophosphamide, the median AUC_E/AUC_P ratios for 4-hydroxycyclophosphamide were 1.8 and 1.6 after oral and intravenous administration respectively [7]. Cyclophosphamide is also more commonly used in solid tumours than in the leukaemias.

The nature of the binding to erythrocytes is currently unclear. Uptake is not restricted to oxazaphosphorine metabolites, and is usually not as high. Many substances bind to erythrocytes [3]; e.g. the concentration of bilirubin [17] and cortisol [18] increases in the erythrocyte compartment once albumin binding sites have been saturated, and red cells act as major transporters of acetaldehyde from the liver to tissues [19].

Therefore it must be appreciated that transport by erythrocytes is the rule rather than the exception [3]. As many different compounds can be transported by red cells, usually weakly bound and with a shorter half-life on erythrocytes compared to plasma [3], a nonspecific binding process such as adsorption to the cell surface comes to mind. The red cell surface area in a human adult is of the order of 3000 km^2, about one million times larger than that of the total capillary endothelium. This ensures that the inner surface of the capillary membrane is in continuous equilibrium with the essentially fragmented membrane of the red cell compartment, which scrapes its surface. However, an association of compounds with proteins in the cell interior, such as that seen during adduct formation between nitrosamine metabolites and globins within haemoglobin [20], cannot be excluded. As far as the transfer of compounds is concerned, the relevant factor is whether those associated with a red cell can be released to the capillary endothelium during passage through the capillary.

In conclusion, ifosfamide and its metabolites, especially isophosphoramide mustard and carboxyifosfamide, are sited primarily on the erythrocyte, which may have a role in the transfer of these compounds from the site of their formation to the tissues. Erythrocyte associated isophosphoramide mustard then acts as a vital transport form of activated ifosfamide.

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Determination of Iphosphamide and Seven Metabolites in Blood Plasma, as Stable Trifluoroacetyl Derivatives, by Electron Capture Chemical Ionization GC-MS

Guido Momerency and Karel Van Cauwenberghe*
Department of Chemistry, University of Antwerp (U.I.A.), B-2610 Antwerp – Wilrijk, Belgium

Ernst A. De Bruijn and Allan T. Van Oosterom
Laboratory for Cancer Research, Department of Medicine, University of Antwerp (U.I.A.), B-2610 Antwerp – Wilrijk, Belgium

Martin S. Highley and Peter G. Harper
Department of Medical Oncology, Guy's Hospital, London SE1 9RT, UK

Key Words:

Oxazaphosphorines
Iphosphamide
Iphosphamide metabolites
Electron capture chemical ionization MS

Summary

A method is described for the determination of the antitumor agent iphosphamide and seven of its metabolites in the plasma of cancer patients by multiple ion monitoring (MIM) GC-MS, mainly using the electron capture chemical ionization mode, of stable methyl and/or trifluoroacetyl derivatives. The metabolites determined were 2- and 3-dechloroethyliphosphamide, 4-ketoiphosphamide, carboxyiphosphamide, iphosphamide mustard, and two previously undetected metabolites, chloroethylamine and 1,3-oxazolidine-2-one.

The isolation of the acidic and neutral metabolites was performed by solid phase extraction on to C_{18} adsorbent at pH 4. The weakly acidic iphosphamide mustard, isolated under these conditions with a yield of *ca* 50 %, was measured as a stable methyltrifluoroacetyl derivative, in contrast to the corresponding phosphoramide mustard of the isomer cyclophosphamide which decomposes during derivatization. Chloroethylamine and 1,3-oxazolidine-2-one were isolated with high yield by liquid extraction with ethyl acetate at pH 10.

Selective measurement of several metabolite derivatives with similar retention times was performed by multiple ion monitoring MS of specific ion masses, using a methyl phenyl siloxane capillary column previously employed in the study of cyclophosphamide metabolites. Quantitation of metabolites in patient plasma samples could be performed in the concentration range 3 ng to 20 μg per ml of original plasma.

1 Introduction

The oxazaphosphorine iphosphamide (IPA; 3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide) is an alkylating antitumor agent; it is structurally different from its isomer cyclophosphamide (CPA) because of a shift of a 2-chloroethyl group from the nitrogen mustard side chain to the nitrogen of the heterocyclic ring [1]. Both IPA and CPA require metabolic activation by hepatic microsomes in order to exhibit cytotoxic activity. The formation of a series of alkylating metabolites and tumor-inactive oxidation products from IPA follows a metabolic scheme similar to that of CPA [1]. The labile primary metabolite, 4-hydroxyiphosphamide, is the precursor of the major cytotoxic compound, the iphosphamide mustard, but can also be further oxidized to the corresponding keto and carboxy derivatives. Two different dechloroethyl derivatives result from oxidation of the side chains with loss of chloroacetaldehyde.

Severe dose-limiting urotoxicity and occasional cases of renal failure initially hindered the development of IPA regimens in cancer chemotherapy. The introduction of concurrent therapy with mercapto ethane sulfonate sodium salt (mesna) [2], to prevent the development of hemorrhagic cystitis by the formation of mesna-conjugation adducts with the primary metabolite 4-hydroxyiphosphamide and acrolein [3], led to a renewed interest in IPA.

Selective and sensitive assays for IPA and its metabolites in biological fluids should be developed by analogy with the CPA system. There are, however, some differences between the behavior of the compounds. Iphosphamide undergoes cyclization in alkaline media resulting in the formation of an aziridine [4]. This reaction lowers the efficiency of extraction of IPA from an alkaline solution, but is totally reversed at acidic pH. IPA itself is sufficiently thermally stable to enable gas chromatographic determination without derivatization [5,6]. In contrast, the isomer cyclophosphamide is perfectly stable under alkaline conditions (no aziridine structure is possible), but needs derivatization to enable GC analysis, otherwise partial thermal cyclization in the injector produces two separate product peaks [5]. Several metabolites of iphosphamide form different derivatives with trifluoroacetic anhydride (TFAA), as will be discussed further below. The trifluoroacetyl derivatives of oxazaphosphorine metabolites can be measured with picogram sensitivity by single ion monitoring (SIM) GC-MS.

This paper presents a full report of procedures for the determination of iphosphamide, the two possible dechloroethyl metabolites, 4-ketoiphosphamide, carboxyiphosphamide, iphosphamide mustard, and the chloroethylamine and oxazolidinone metabolites in blood plasma, using electron capture chemical ionization and electron impact mass spectrometric detection of the trifluoroacetyl derivatives. The procedure for the determination of CPA metabolites in blood plasma by similar methods has been described elsewhere [7].

2 Experimental

2.1 Chemicals and Extraction Procedure

Reference compounds of most of the metabolites measured in this study were generously provided by ASTA-Werke, Degussa Pharma Gruppe, Bielefeld, Germany. Water and organic solvents used in the procedure were of HPLC quality grade. The trifluoroacetic anhydride reagent (TFAA) was obtained from Janssen Chimica, Geel,

Belgium. pH 4 buffer was a 0.05 M citrate – hydrochloric acid mixture. SEP-PAK solid phase extraction cartridges containing 500 mg of trifunctional C_{18} reversed phase material, supplied by Millipore, were used on a vacuum manifold station [7].

Details of the enrichment procedure have been described elsewhere [7]. Original plasma samples were diluted with pH 4 citrate buffer and the extraction of the major metabolites performed using C_{18} cartridges. After elution with methanol and evaporation under nitrogen, derivatization was accomplished by reaction with diazomethane in ether and trifluoroacetic anhydride in ethyl acetate. The newly identified chloroethylamine and 1,3-oxazolidine-2-one metabolites were isolated by ethyl acetate extraction after dilution with 0.05 M borate – sodium hydroxide buffer of pH 10.

2.2 Chromatography and Mass Spectrometry

Gas chromatographic separations were performed on a Carlo Erba Mega Series 5160 chromatograph equipped with a 25 m × 0.25 mm i.d. fused silica capillary column coated with a 0.2 μm film of Sil-13 (methyl phenyl siloxane; Chrompack, Middelburg, Holland). Both split (1:10) and splitless (45 s) injection were performed; the injector temperature was 250 °C.

The fused silica GC column was introduced, through a short metal capillary transfer line kept at 250 °C, into the combined electron impact – chemical ionization source of a Fisons Trio-2A quadrupole mass spectrometer (Fisons Instruments UK) maintained at 180 °C. Electron impact (EI) ionization was performed with a trap current of 200 μA, an electron energy of 70 eV, and an ion repeller potential of 1 V. Chemical ionization (CI) was performed with a source current of 800 μA, an electron energy of 40 eV, and an ion repeller potential of 0 V. Multiple ion monitoring was performed on extracts of spiked plasma and patient plasma. Linear temperature programming of the GC column enabled selection of retention windows in the chromatogram during which specific *m/z* values from the mass spectra of the metabolites were measured.

2.3 Syntheses of the Two New Metabolites

2.3.1 Chloroethylamine

Sodium hydroxide (0.5 g; 12.5 mmol) was added to a solution of chloroethylamine hydrochloride (1.15 g; 10 mmol) in water (20 ml). This mixture was stirred for 2 min and extracted with ethyl acetate (5 × 10 ml). This extract was dried over anhydrous sodium sulfate for 24 h. After evaporation of the solvent the chloroethylamine was isolated as a colorless fluid.

2.3.2 1,3-Oxazolidine-2-one

Chloroethylamine hydrochloride (1.15 g; 10 mmol) was added to a solution of sodium bicarbonate (0.84 g; 10 mmol) and sodium hydroxide (0.4 g; 10 mmol) in 30 ml water. The mixture was kept at 40 °C for 45 min, then extracted with ethyl acetate (5 × 10 ml). The extract was dried over anhydrous sodium sulfate and the 1,3-oxazolidine-2-one isolated as a pale yellowish fluid after evaporation of the solvent. GC-MS analysis of a solution of this residue revealed the presence of < 2 % of the original chloroethylamine in the extract.

3 Results and Discussion

3.1 Mass Spectra of Trifluoroacetylated Metabolites of IPA

The mass spectra of the trifluoroacetylated parent compounds IPA and CPA have been published elsewhere [7]. Trifluoroacetyliphosphamide produces a positive electron impact mass spectrum similar to that of the cyclophosphamide derivative, with a base peak at *m/z* = 307/309 for the fragment $(M - CH_2Cl)^+$, as first reported by *Lartigue-Mattei et al.* [8]. The characteristics of the EI+ and CI– mass spectra of the derivatives of the metabolites measured in this study are presented in **Table 1**.

Table 1
Characteristic fragments of the EI+ and CI– mass spectra of IPA metabolite derivatives.

Compound	M.W.	Fragment	Interpretation
EI+			
TFA-IPA	356	307/309	$(M - CH_2Cl)^+$
TFA-CPA (IS)	356	307/309	$(M - CH_2Cl)^+$
TFA-2-DCEI	294	245	$(M - CH_2Cl)^+$
bis TFA-3-DCEI	390	341	$(M - CH_2Cl)^+$
TFA-Carboxy-IPA-Me	402	324/326	$(M - NHCH_2CH_2Cl)^+$
TFA-Keto-IPA	370	335/337	$(M - Cl)^+$
TFA-CEA	175	126	$(M - CH_2Cl)^+$
TFA-OXAZ	183	114	$(M - CF_3)^+$
TFA-IPM-Me	330	281/283	$(M - CH_2Cl)^+$
CI–			
Group I			
TFA-CPA	356	320/322	$(M - HCl)^-$
TFA-2-DCEI	294	274/276	$(M - HF)^-$
TFA-OXAZ	183	183	$(M)^-$
TFA-Carboxy-IPA-Me	402	185	?
Group II			
TFA-IPA	356	293/295	$(M - CH_2CH_2Cl)^-$
bis TFA-3-DCEI	390	327	$(M - CH_2CH_2Cl)^-$
TFA-Keto-IPA	370	307/309	$(M - CH_2CH_2Cl)^-$
TFA-CEA	175	112	$(M - CH_2CH_2Cl)^-$
TFA-IPM-Me	330	267/269	$(M - CH_2CH_2Cl)^-$

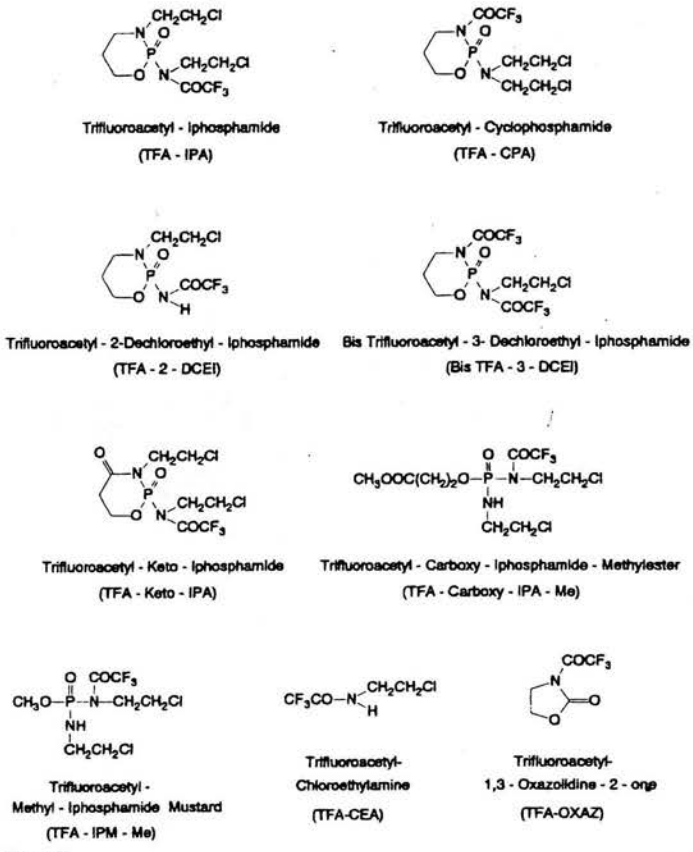

Figure 1
Structures of the stable metabolite derivatives of iphosphamide.

The structures of the metabolite derivatives, their full names, and the abbreviations used in this paper are given in **Figure 1**.

Selected EI+ and Cl– mass spectra of metabolite derivatives are shown in **Figures 2** and **3**.

The metabolite 2-DCEI is produced only by the metabolism of IPA (not of CPA) 3-DCEI is identical with the dechloroethyl metabolite of cyclophosphamide and is therefore a metabolite of both oxazaphosphorines. After derivatization with TFAA, the 2-DCEI was, therefore, measured as the monotrifluoroacetyl derivative and the 3-DCEI metabolite as the bistrifluoroacetyl derivative (**Figure 4**). The mass spectrum of the latter compound was given in a previous paper [7]. No bistrifluoroacetyl derivative of 2-DCEI was ever detected.

Carboxyiphosphamide has two identical phosphoramidic functions which can be further substituted. Theoretically both can be trifluoroacetylated, but in practice only one is actually transformed, probably because of the effect of steric hindrance in the mono derivative. In the corresponding carboxy metabolite of cyclophosphamide, a further loss of hydrogen chloride is observed yielding a single cyclic derivative as the only chromatographic peak [7]. A similar process did not occur with the carboxyiphosphamide. The structures of the final derivatives of the two corresponding carboxy metabolites of IPA and CPA are thus different, as is shown in **Figure 5**.

In the procedure used to measure the stable cyclophosphamide metabolites in blood plasma [7], it was observed that negative chemical ionization in the electron capture mode, using methane as quenching gas, provided much better sensitivity than electron

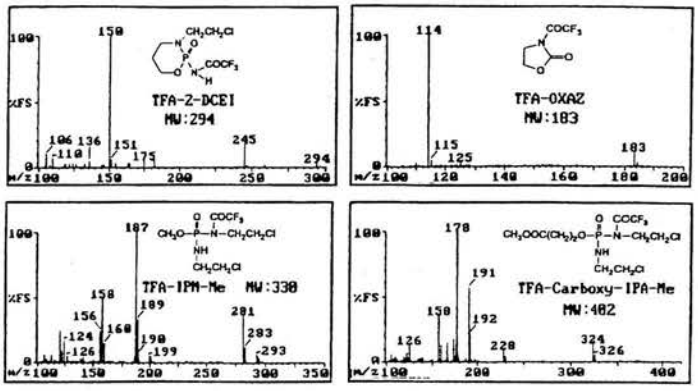

Figure 2
Electron impact mass spectra of TFA-2-DCEI, TFA-1,3-oxazolidine-2-one. TFA-IPM-Me, and TFA-Carboxy-IPA-Me.

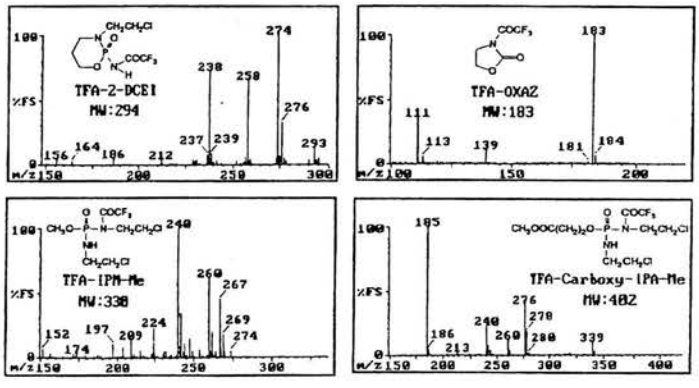

Figure 3
Electron capture chemical ionization mass spectra of TFA-2-DCEI, TFA-1,3-oxazolidine-2-one, TFA-IPM-ME, and TFA-Carboxy-IPA-Me.

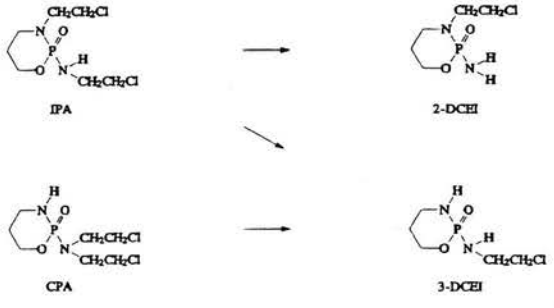

Figure 4
The formation of the dechloroethyl metabolites of IPA and CPA.

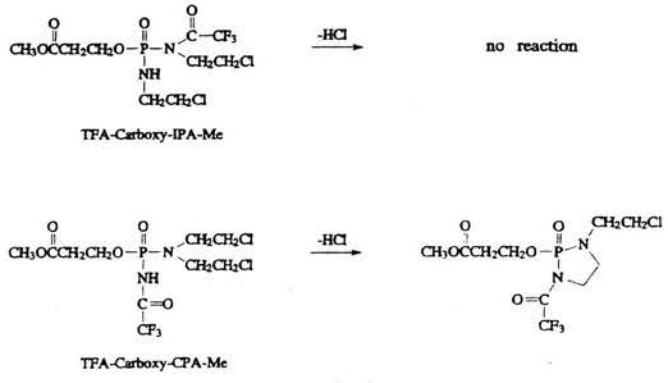

Figure 5
The structures of the carboxy derivatives of IPA and CPA.

impact or positive chemical ionization for many trifluoroacetylated metabolite derivatives. The same is true for the IPA metabolites.

In electron capture (CI–) mode, two different types of fragmentation occur for the IPA and the CPA metabolites; this is illustrated by subdivisions I and II in Table 1. The derivatives in group II contain a special functional group which fragments by the loss of a CH_2CH_2Cl substituent, *e.g.* the iphosphamide mustard derivative with a characteristic peak at *m/z* 267/269. A tentative mechanism is shown in **Figure 6**

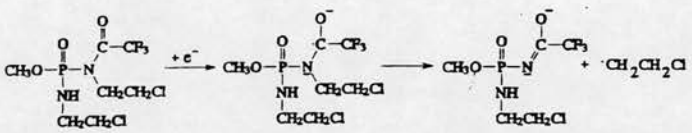

Figure 6
Fragmentation of the derivative of IPM in electron capture chemical ionization MS.

A tentative assignment for the base peak fragment ion from the carboxy-IPA derivative at *m/z* = 185 cannot yet be proposed. The fragment is, however, of diagnostic value for the carboxy-IPA derivative; it is, therefore, further used in quantitative work.

3.2 The Derivatization Step

The derivatization procedure applied to IPA metabolites isolated from plasma is identical to that for CPA metabolites described elsewhere and involves two steps – methylation of acidic functions of carboxyiphosphamide and iphosphamide mustard followed by trifluoroacetylation of unhindered phosphoramide functions [7] using a large excess of the reagents. The bis-trifluoroacetyl derivative of the 3-DCEI metabolite is produced quantitatively when, after methylation of the plasma extract by diazomethane in ether, the solvent is evaporated completely and replaced by ethyl acetate for trifluoroacetylation. On standing in the presence of ether or chloroform, an equilibrium mixture of the two mono derivatives of 3-DCEI is formed from the bis derivative.

The metabolite 4-ketocyclophosphamide, cannot be derivatized by reaction with TFAA because of a mesomeric effect involving the endocyclic amidic nitrogen atom [7]. In contrast, trifluoroacetylation of 4-ketoiphosphamide occurs smoothly on the exocyclic nitrogen atom. 4-Ketoiphosphamide does not react with diazomethane solution. Unlike the cyclophosphamide mustard (CPM), which decomposes completely to the nor-nitrogen mustard during attempts to derivatize with TFAA, the iphosphamide mustard (IPM) does not decompose during the enrichment and derivatization steps and can easily be recovered from plasma. Methylation occurs only on the phosphoramidic acid function and trifluoroacetylation only on one of the equivalent phosphoramidic nitrogen atoms (**Figure 7**). The derivative has an acyclic structure and the subsequent loss of hydrogen chloride with the formation of a cyclic product, seen with several CPA metabolites [7], is not observed with this derivative.

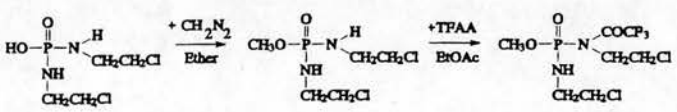

Figure 7
Derivatization of IPM.

3.3 Recovery of IPA Metabolites from Spiked Plasma Samples

An extensive study was performed to determine the efficiencies of extraction of mixtures of CPA and its metabolites from plasma samples spiked at typical concentrations of 10 µg/ml, using liquid extraction with ethyl acetate or chloroform and solid phase isolation with either silica gel or C_{18} at pH 4, 7, or 10. The results of these experiments have previously been discussed in detail [7]. In view of the similar recoveries obtained for IPA metabolites under these conditions, an extensive study at these pH values in triplicate for all methods of isolation was not performed. The only comparison was of the extraction yields obtained for CPA and IPA metabolites using the C_{18} solid phase extraction procedure at acidic pH. The results of these experiments are listed in **Table 2**.

Table 2
Recoveries of IPA and CPA metabolites using C_{18} solid phase extraction at pH4.

CPA	IPA
CPA: 93.2 % ± 3.6 %	CPA (IS): 93.2 % ± 3.6 %
IPA (IS): 99.7 % ± 3.7 %	IPA: 99.7 % ± 3.7 %
DCEC: 99.5 % ± 2.8 %	3-DCEI 96.8 % ± 4.1 %
	2-DCEI 92.7 % ± 6.4 %
Keto-CPA: 90.3 % ± 2.6 %	Keto-IPA: 91.5 % ± 3.8 %
Carboxy-CPA: 82.5 % ± 3.9 %	Carboxy-IPA: 74.4 % ± 1.9 %
Alco-CPA[a]: 75.9 % ± 5.0 %	–
CPM[b]: 56.3 % ± 4.0 %	IPM: 49.8 % ± 4.2 %

[a] Alco-IPA was not available as a reference compound.
[b] Measured by thermospray LC-MS.

The observed extraction efficiencies at pH 4 are generally higher than 90 % for both dechloroethyl metabolites, the keto metabolite, and for the parent compounds IPA and CPA. The recovery of the carboxy metabolite of IPA is somewhat lower, *ca* 75 %. Both iphosphamide and cyclophosphamide mustard are stable in a weakly acidic medium. As the cyclophosphamide mustard decomposed readily during attempts at derivatization, the efficiency with which it was extracted was measured by thermospray LC-MS (single ion monitoring) of the underivatized form of the molecule. The ion monitored was the $(M + NH_4 - HCl)^+$ at *m/z* 202. The recovery of the iphosphamide mustard from C_{18} columns is low, only about 50 %, and comparable with that of CPM, but still 10 % higher than the value given in the literature for extraction of CPM using Amberlite XAD-4 columns [9].

The isolation of the alkaline chloroethylamine and 1,3-oxazolidine-2-one metabolites was performed by liquid extraction with ethyl acetate at pH 10; recoveries were 85 % and 90 % respectively, an improvement on the solid phase procedure at pH 7. No further decomposition of the metabolites was observed during storage of spiked plasma samples at –20 °C for at least three weeks. The schematic diagram in **Table 3** illustrates the enrichment procedure applied to patient plasma samples.

3.4 Gas Chromatographic – Mass Spectrometric Analysis of the Metabolites

From the study of the gas chromatography of the CPA metabolites, it was found that complete separation of all the metabolites could be achieved on a 25 m × 0.25 mm i.d. capillary column coated with a 0.2 µm film of Sil-13 (Chrompack) [7]. The same capillary column

Table 3
Schematic diagram of the enrichment procedure.

Determination of IPA, Keto-IPA, IPM, Carboxy-IPA, and 2- and 3-DCEI	Determination of CEA and OXAZ
0.5 ml plasma	0.5 ml plasma
↓ + internal standard CPA	↓ + internal standard CEOX
+ 0.5 ml buffer pH 4	+ 0.5 ml buffer pH 10
↓	↓
1 ml diluted plasma, vortex	1 ml diluted plasma, vortex
↓	↓
C_{18} column (500 mg)	extraction with ethyl acetate
↓	(3 ml + 2 ml), centrifugation
elution with 5 ml methanol;	↓
complete evaporation	partial evaporation
↓	↓
dry residue	derivatization
+ diazomethane in ether for 15 min	+ 0.3 ml ethyl acetate
evaporation	+ 0.3 ml TFAA at 70 °C for 2h
↓	↓
derivatization	partial evaporation
+ 0.3 ml ethyl acetate	↓
+ 0.3 ml TFAA at 70 °C for 2h	
↓	
complete evaporation	dissolve in 250 µl ethyl acetate
dissolve in 50 µl ethyl acetate	↓
↓	
GC-MS	GC-MS

Table 4
Retention data for CPA and IPA metabolites on Chrompack Sil-13[a].

CPA	t_R [min]	IPA	t_R [min]
bis TFA-DCEC	7.41	TFA-IPM-Me	6.71
TFA-IPA (IS)	12.66	bis-TFA-3-DCEI	7.40
bis TFA-Alco-CPA	13.88	TFA-2-DCEI	8.26
TFA-CPA	14.82		
TFA-Carboxy-CPA-Me	17.48	TFA-IPA	12.63
Me-Keto-CPA	18.25	TFA-Keto-IPA	12.93
		TFA-Carboxy-IPA-Me	14.03
		TFA-CPA (IS)	14.83

[a] Linear temperature program from 150 to 240 °C at 6 °/min.

was, therefore, used for the separation of the derivatives of the IPA metabolites. **Table 4** lists the retention times of the metabolites of both oxazaphosphorine derivatives on this column in order of increasing retention time.

It is apparent from the table that separate retention windows can easily be established for single ion monitoring of the individual metabolites of cyclophosphamide. Since two regions of partial overlap exist for iphosphamide metabolites, in which retention times differ by less than 1 min, multiple ion monitoring was applied in these regions, giving complete separation using the appropriate specific ion masses, although the ultimate detection sensitivity was reduced by a factor of 2 to 3. Splitless injection of a 1 µl aliquot from 50 µl of final derivatized extract enabled determination of metabolite plasma concentrations in the range of 3 ng/ml to 20 µg/ml.

Table 5
Multiple ion monitoring programs for IPA metabolites[a].

Metabolite derivative	Specific ion	*m/z*	Retention window [min]
CI-			
TFA-IPM-Me	$(M - CH_2CH_2Cl)^-$	266.99	2.0–10.0
bis TFA-3-DCEI	$(M - CH_2CH_2Cl)^-$	327.00	2.0–10.0
TFA-2-DCEI	$(M - HF)^-$	274.00	2.0–10.0
TFA-IPA	$(M - CH_2CH_2Cl)^-$	293.01	10.0–13.5
TFA-Keto-IPA	$(M - CH_2CH_2Cl)^-$	306.99	10.0–13.5
TFA-Carboxy-IPA-Me	$(M - ?)^-$	185.01	13.5–17.0
TFA-CPA (IS)	$(M - HCl)^-$	320.03	13.5–17.0
EI+			
TFA-CEA	$(M - CH_2Cl)^+$	126.02	2.0– 6.0
TFA-OXAZ	$(M - CF_3)^+$	114.02	6.0–12.0
CEOX (IS)	$(M - CH_2Cl)^+$	100.04	6.0–12.0

[a] Dwell time 0.08 s, span 0.02 a.m.u.

The multiple ion monitoring time intervals in order of increasing retention times were as listed in **Table 5**.

Negative chemical ionization by electron capture was the most sensitive technique for the quantitative measurement of IPA, 3-DCEI, 4-keto-IPA, and carboxy-IPA; no substantial difference was observed between EI+ and CI– sensitivity for 2-DCEI and IPM. These metabolites can, therefore, all be measured with equally high sensitivity by electron capture CI– (a typical MIM chromatogram is

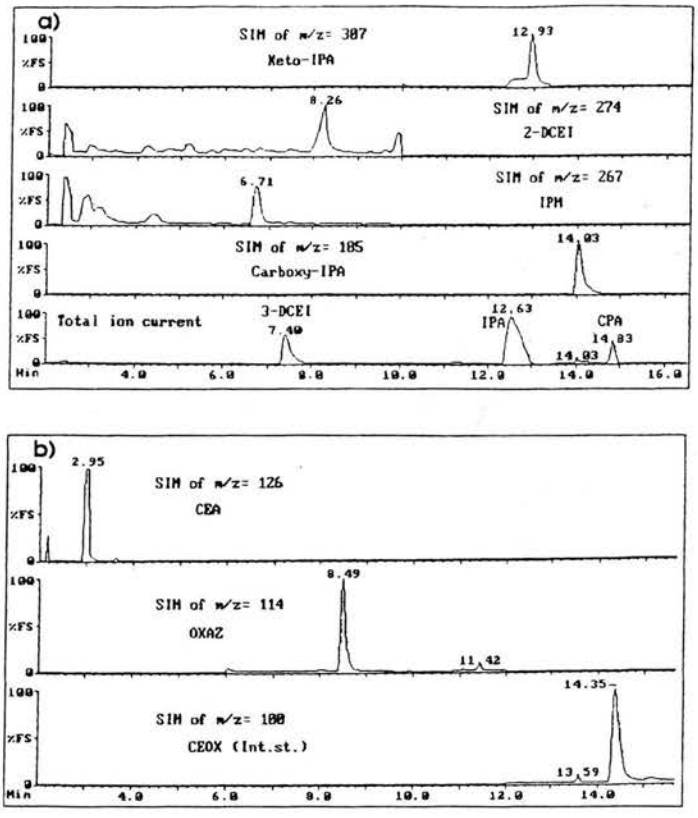

Figure 8
CI– MIM chromatogram (a) and EI+ SIM chromatogram (b) for a patient plasma extract.

shown in **Figure 8a**). Only the CEA and OXAZ metabolites were measured with greater sensitivity by electron impact ionization, using a temperature program from 80 to 150 °C at a rate of 6 °/min (a typical SIM chromatogram is shown in **Figure 8b**).

To compensate for differences in extraction efficiency and chromatographic variations after derivatization, CPA was added to the original plasma as internal standard (IS) for quantification of most IPA metabolites. A different internal standard (the corresponding *N*-chloroethyl-1,3-oxazolidine-2-one metabolite of CPA (CEOX), available as a reference compound) was used for the measurement of the chloroethylamine and 1,3-oxazolidine-2-one metabolites. Blank plasma samples spiked with a mixture of metabolites were submitted to the same enrichment procedure as the patient samples, and the sample extracts used after derivatization to construct calibration curves in the concentration ranges 5 to 100 ng and 0.1 to 20 µg metabolite per ml of original plasma. Measurements of patient and spiked plasma sample extracts were, where possible, performed on the same day, in order that calibration curves and patient plasma measurements were determined under similar conditions of ion source contamination.

Linear regression analysis of the measured data points (ratio of metabolite peak area to internal standard peak area, Y, against metabolite concentration, X, in the original plasma) produces a best fit; as an example, the regression equation for IPM was $Y = (0.01579 \pm 0.00037)X + (0.00125 \pm 0.00022)$ ($n = 6$)

The intra-assay reproducibility, expressed as relative standard deviation, was approximately 3 %, and the inter-assay reproducibility ranged from 5 to 10 % for sets of four independent extractions.

Measurement of the labile primary metabolite 4-hydroxyiphosphamide as a separate peak was not possible by the procedure described herein. Since the hydroxy structure is stabilized at a weakly acidic pH (*Ikeuchi et al.* [10]) and, at least in the case of the isomer cyclophosphamide, will easily decompose to the phosphoramide mustard during the C_{18} solid phase extraction procedure (*Watson et al.* [11]), we suggest that the measured iphosphamide mustard concentration actually represents the sum of the free mustard and the hydroxyiphosphamide and aldophosphamide in the patient plasma samples. This hypothesis will be confirmed by additional experiments, using the recent method of *Hong* and *Chan* [12] to measure the original hydroxy – aldo tautomeric pair as a silylated cyanohydrin after liquid extraction of the plasma.

The proposed method of analysis has been applied successfully to the determination of iphosphamide metabolites in the blood plasma of patients after oral administration of iphosphamide in combination with mesna (IPA/Mesna chemotherapy).

Figure 9 shows a typical concentration – time profile of IPA-metabolites from a cancer patient who received an oral dose of 500 mg iphosphamide in combination with mesna.

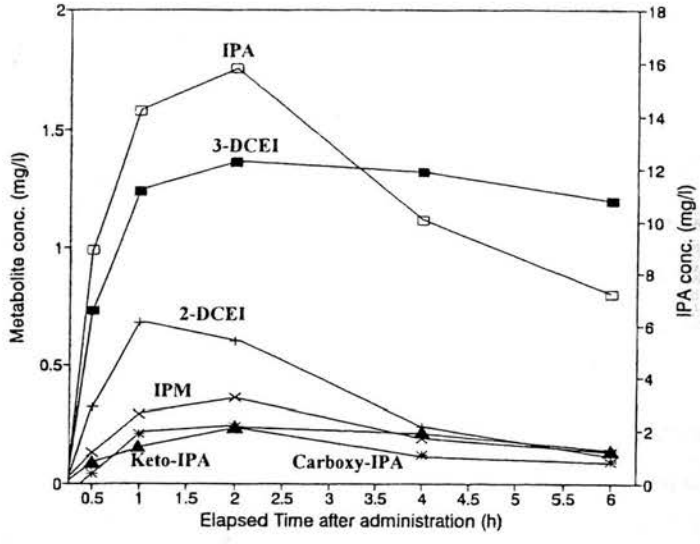

Figure 9
Example of a profile of concentration against time for IPA metabolites in the blood plasma of a cancer patient.

The interpretation of the pharmacokinetic results of our studies will be discussed elsewhere.

4 Conclusion

A new analytical method is presented for the determination of the antitumor drug iphosphamide and seven metabolites in the blood plasma of cancer patients. After solid phase extraction on to C_{18} columns and reaction of the residue with diazomethane and trifluoroacetic anhydride, the stable derivatives were separated on a methyl phenyl siloxane GC capillary column and measured selectively by multiple ion monitoring GC-MS. The acidic and neutral metabolites were measured by electron capture chemical ionization. The active iphosphamide mustard was determined as a stable methyltrifluoroacetyl derivative. The labile primary metabolite 4-hydroxyiphosphamide could not be determined by this procedure and will require trapping in the original plasma.

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