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The effect of route of administration and fractionation of dose on the metabolism of ifosfamide

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Summary. The measurement of urinary ifosfamide, isophosphoramide mustard, dechloroethyl ifosfamide and carboxyifosfamide using high-performance thin-layer chromatography with photographic densitometry (TLC-PD) is described. This technique was also used to demonstrate the large inter-individual variation in the ifosfamide metabolic profile of patients receiving the drug as single-agent therapy for non-small-cell lung cancer. In addition, oral administration was shown to result in higher levels of these metabolites in the urine. Fractionation of the ifosfamide dose over several days resulted in increasing levels of metabolites in the urine, consistent with auto-induction of ifosfamide metabolism.

Introduction

Ifosfamide [*N*,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide, Mitoxana] is a structural isomer of the oxazaphosphorine cyclophosphamide. Ifosfamide differs from cyclophosphamide in that one of the chloroethyl side chains is transposed from the exocyclic nitrogen of the phosphoramidic mustard group to the endocyclic nitrogen of the oxazaphosphorine ring. Like cyclophosphamide, it is a prodrug that requires biotransformation to become cytotoxic. This occurs mainly in the liver [2] by the action of cytochrome P450, producing 4-hydroxyifosfamide [5], which then can be oxidised to 4-ketoifosfamide, an inactive metabolite, or, alternatively, can tautomerise to produce aldoifosfamide. Aldoifosfamide can in turn undergo either dehydrogenation by the action of aldehyde dehydrogenase to carboxyifosfamide or spontaneous β-elimination of acrolein to yield isophosphoramide mustard, which is thought to be the ultimate alkylating agent. Ifosfamide metabolism is known to differ from that of cyclophosphamide in that, in addition to the pathway described above, *N*-dealkylation of the side chain can occur, resulting in the production of dechloroethyl metabolites (either dechloroethyl ifosfamide or cyclophosphamide) and chloroacetaldehyde [16] (see Fig. 1). Chloroacetaldehyde production has recently been implicated in the aetiology of the neurotoxic syndrome that is occasionally seen following ifosfamide administration but never following cyclophosphamide use [8].

Methods of measuring oxazaphosphorine metabolites have traditionally relied on complex and time-consuming technologies such as gas chromatography-mass spectroscopy. This paper describes a simpler technique for measuring urinary excretion of ifosfamide and its metabolites, isophosphoramide mustard, dechloroethyl ifosfamide plus dechloroethyl cyclophosphamide (referred to hereafter collectively as dechloroethyl ifosfamide) and carboxyifosfamide. This technique was used to determine the inter-patient variability of ifosfamide metabolism and the effect of the route of administration and schedule on ifosfamide disposition.

Patients and methods

Patients. A total of 20 patients with advanced histologically proven non-small-cell lung cancer were treated with single-agent ifosfamide. Their median age was 59.5 years (range, 44–71 years), and all had pretreatment Karnofsky scores of >50 and creatinine clearances of >50 ml/min. Two regimens were used, as follows:

1. Ten patients received i.v. ifosfamide (Boehringer Ingelheim, Mitoxana) at a dose of 1.5 g/m^2 per day for 5 days. The dose was rounded down to the nearest 500 mg, and ifosfamide was given as a 30-min infusion in 250 ml normal saline. The uroprotector 2-mercaptoethane sodium (mesna) was given at a dose of 1.5 g/m^2 per day in 2 l normal saline as a 24-h infusion for 5 days.

2. Ten patients received oral ifosfamide (only available as 500-mg capsules), 30–60 min before breakfast at a dose of 1.5 g/m^2 per day for 5 days. The dose was rounded up or down as outlined in regimen 1. Mesna was given in a manner similar to that outlined in regimen 1, with

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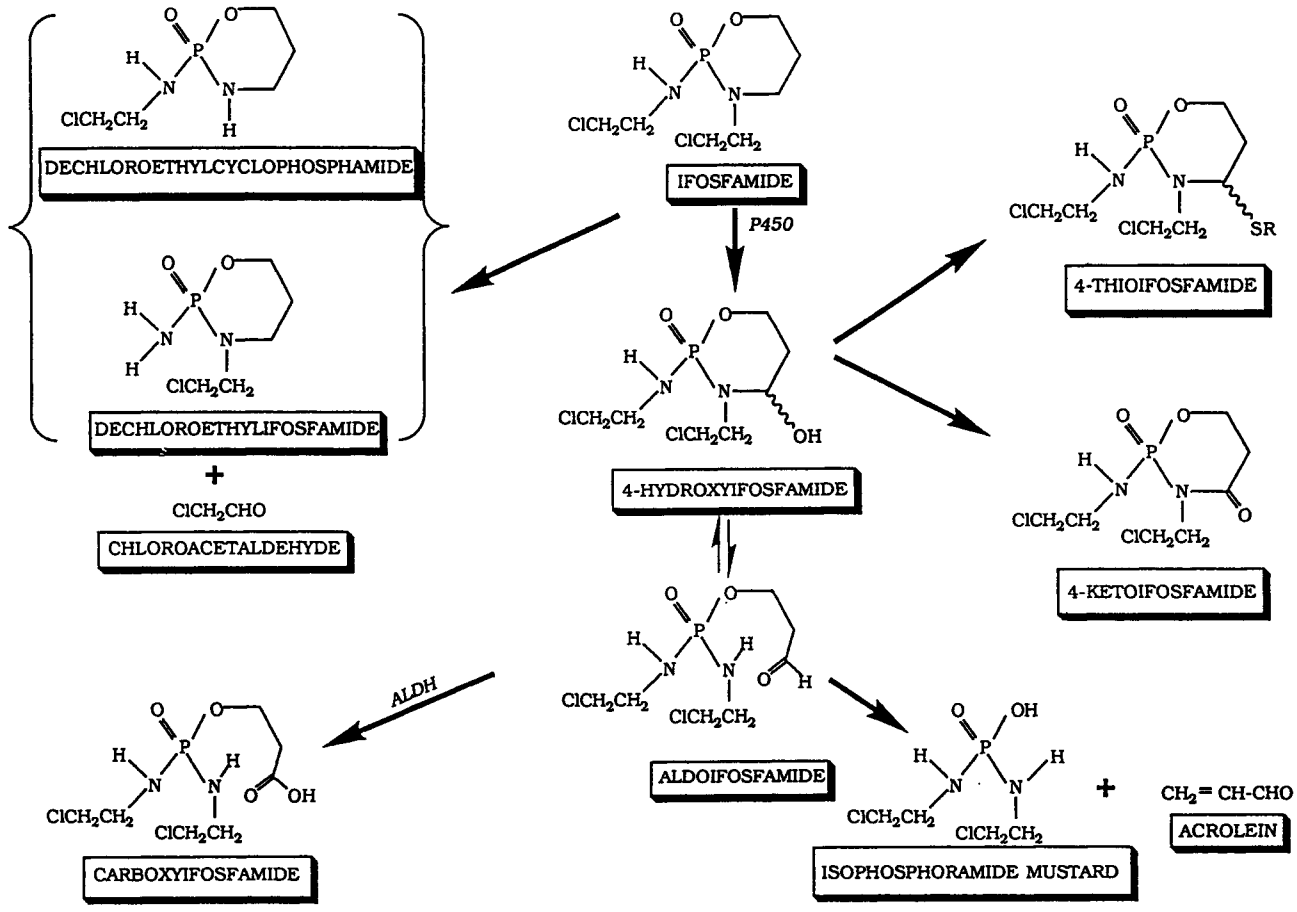

Fig. 1. Metabolic pathways of ifosfamide

a further 500 mg given p.o. 6 h for 4 doses after the last i.v. mesna dose on day 5.

Sample collection. Urine samples were collected at 24-h intervals for each day of ifosfamide administration and aliquots were immediately frozen and stored at –20° C.

Materials. Ifosfamide, dechloroethyl ifosfamide, isophosphoramide mustard and carboxyifosfamide were all kindly donated by Degussa (Bielefeld, FRG). Nitrobenzyl pyridine (NBP) was obtained from the Sigma Chemical Company (Poole, Dorset, UK). Amberlite XAD-2 non-anionic polymeric adsorbent were obtained from the Aldrich Chemical Company (Gillingham, Dorset, UK) and packed in glass pasteur pipettes (8 × 0.6 cm). All other reagents were Analar-grade. Precoated aluminium-backed silica gel 60 F_{254} (20 × 20 cm) high-performance thin-layer chromatographic (HPTLC) plates were supplied by Merck (Darmstadt, FRG). HPTLC plates were photographed using Ilford FP4 125 ASA film. Densitometric quantitation was carried out using a dual-wavelength thin-layer chromatographic scanner (Shimadzu model CS-930; Dyson Instruments Ltd, Houghton-le-Spring, Tyne and Wear, UK) fitted with a Shimadzu DR-2 data recorder.

Thin-layer chromatography-photographic densitometry. Thin-layer chromatography with photographic densitometry (TLC-PD) was carried out according to the method of Hadidi and Idle [9]. Samples were treated as follows: urine (1 ml) was applied to the XAD-2 columns, which had been pre-washed with successive volumes of acetone (3 ml), methanol (3 ml) and water (3 ml) to remove monomers and inorganic salts. Following elution of the urine samples, the columns were washed with glass-distilled water (3 ml) and sucked dry with a water pump. Subsequently, ifosfamide and its metabolites were eluted with methanol (5 ml); the resulting eluent was reduced to dryness in vacuo at 40° C using a Savant Speedvac evaporating centrifuge (V. A. Howe and Company Ltd, Croydon, UK). The dry residues were then reconstituted in 30 µl methanol and applied to the HPTLC plates, which had been pre-eluted with methanol and dried at 150° C for 2× 10 min.

Chromatography was carried out in glass TLC tanks saturated with solvent, using chloroform, ethanol and glacial acetic acid (100:20:1, by vol.) as the mobile phase and run to a height of at least 18 cm. The plates were removed and dried in cold air. Subsequently, they were sprayed with 5% 4-(4-nitrobenzyl)pyridine (NBP) in acetone mixed with 0.2 *M* acetate buffer (pH 4.6) (8:2 v/v) for at least 15 s, dried again in cold air and resprayed for a further 15 s with the NBP/acetate buffer mixture. The plates were once more dried in cold air and then heated in an oven at 130°–150° C for 5–15 min. After cooling, they were dipped in a 3% methanolic potassium hydroxide solution, where upon characteristic blue spots developed; as these are extremely unstable, the plates were photographed within 10 s of development of the spots.

The plates were photographed 30 cm below the film plane of a 5 × 4-in. bellows camera with two tungsten halogen 1.275-kW lights, which illuminated the plates at 45° C from each side (plates were photographed using a 150-mm lens stopped down to f11 with a one-eighth of a second exposure onto Ilford FP4 125 ASA film). To ensure uniform exposure, each plate was photographed together with a Kodak grey scale; once exposed, the negative was developed for 2 min at 20° C in Ilford contrast FF diluted five-fold. The negative was subsequently rinsed in an acetic acid stop bath and then fixed, washed and dried. The negative was enlarged using a 150-mm lens stopped down to f22 to exactly 1 magnification of the original TLC plate for approximately 7 s. Kentmere resin-coated glossy paper (grade 2) was used and then processed for 1 min at 20° C with Ilfospeed diluted ten-fold.

For densitometric estimation, photographs were mounted on uncoated glass thin-layer chromatographic plates. Chromatographic lanes were scanned with a wavelength of 370–600 nm in the reflection mode with background subtraction and using a light spot of 1 × 2 mm. Care was taken to ensure that the zig-zag excursion of the light spot encom-

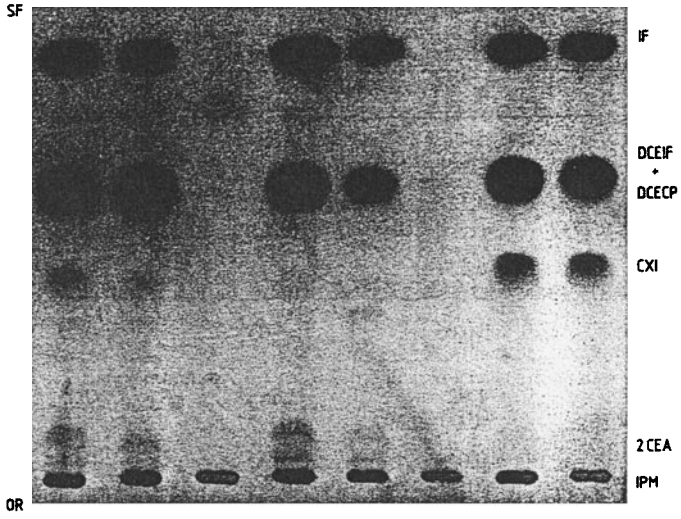

Fig. 2. Photograph of an HPTLC plate developed in chloroform: ethanol: acetic acid (100:20:1, by vol.) and visualised with 3% NBP

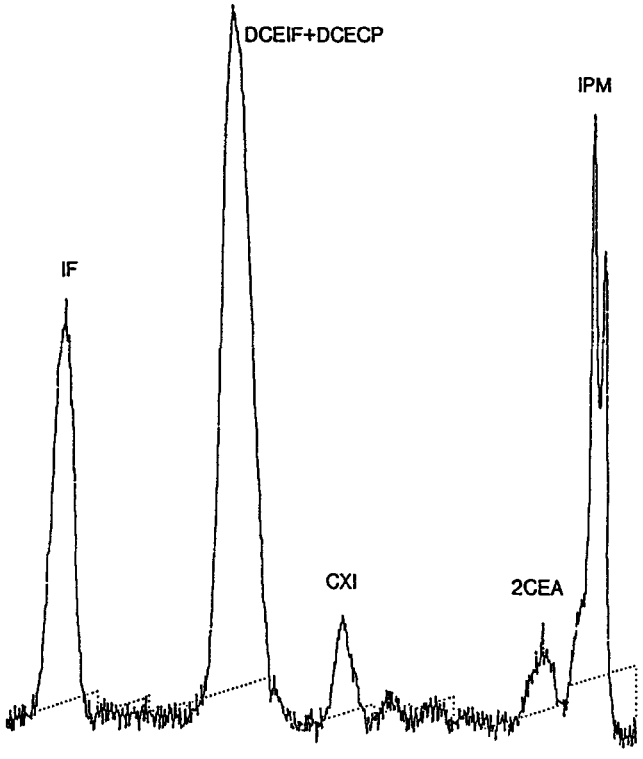

Fig. 3. Resolution of ifosfamide metabolites in human urine by TLC-PD. *IF*, ifosfamide; *DCEIF+DCECP*, dechloroethyl ifosfamide + dechloroethyl cyclophosphamide; *CXI*, carboxyifosfamide; *2CEA*, ●; *IPM*, isophosphoramide mustard

passed each complete chromatographic spot. Chromatograms were plotted at low sensitivity and peak areas calculated by the data recorder. Standard curves were constructed by spiking blank urine with the metabolites over the range of 1–250 μg/ml.

Statistical methods. To determine any change in metabolite excretion with time a Friedman two-way analysis of variance was used; to compare parameters between the i.v. and oral groups, a Mann-Whitney test was applied. Kendall rank-correlation tests were used employed to examine possible correlations between metabolite levels and toxicity.

Results

Clinical data

Toxicity. The median nadir white blood cell count (measured 10 days from the start of chemotherapy) experienced in the i.v. group was 3.8×10^9 cells/l and that in the oral group, 5.45×10^9 cells/l ($P > 0.05$, Student's *t*-test). Five of the patients in the oral arm experienced CNS toxicity greater than WHO grade III [15], requiring early termination of their treatment course, as opposed to none of the patients in the i.v. arm ($0.01 < P < 0.02$, chi-square test). Two of the patients in the oral arm became comatosed for 24–48 h but recovered completely, with no neurological sequelae. Mild degrees of encephalopathy, consisting mainly of somnolence (<50% of waking hours), were seen in the i.v. arm of the study. Nausea and vomiting were mild and identical in both arms.

Tumour response. A total of eight partial responses were seen, four occurring in each arm.

Ifosfamide metabolites

A representive chromatogram is shown in Fig. 2 and a representive densitometric scan, in Fig. 3. The R*f* values for ifosfamide and its metabolites were as follows: ifosfamide, 0.70; dechloroethyl compounds, 0.47; carboxyifosfamide, 0.33; and isophosphoramide mustard, 0.02. The calibration curves were linear over the range used. The results of urinary analyses are shown in Table 1. The percentage of the delivered ifosfamide dose recovered from the urine as alkylating metabolites (isophosphoramide mustard, dechloroethyl ifosfamide and carboxyifosfamide) was low, with a median value of 6.5% (range, 1.18%–45.72%) obtained on day 1 for both the i.v. and oral groups. The median value for the total alkylating metabolite excretion for the i.v. group was 3.45% (range, 1.18%–7.8%), which was statistically significantly lower than that for the oral group [15.18% (range, 1.88%–32.7%); $P < 0.05$, Mann-Whitney test]. On the remaining days, total alkylating metabolite excretion was always greater in the oral group than in the i.v. group, but the values did not attain statistical significance. Over the 5-day period, total alkylating metabolite excretion in the i.v. group increased from the above median value to that of 8.13% (range, 0.92%–59.95%) on day 5 ($P \approx 0.05$, Friedman test), and in the p.o. group it increased from the mean value on day 1 to that of 29.15% (range, 2.20%–81.64%) on day 3 ($P < 0.05$, Friedman test).

The most abundant of these alkylating urinary metabolites was dechloroethyl ifosfamide, with a median excretion value of 3.56% (range, 0.61%–26.63%) on day 1 for both the i.v. and oral groups. From Table 1 it can been seen that dechloroethyl ifosfamide excretion was subject to a high degree of inter-patient variability. Oral administration resulted in statistically higher urinary levels than did i.v. application on the 1st day of ifosfamide treatment ($P < 0.05$, Mann-Whitney test); urinary excretion remained higher on the remaining days in the oral group, but the

Table 1. Pattern of ifosfamide metabolite excretion
Values are medium percentages of dose administered; values in brackets are ranges

DAY		1	2	3	4	5	P*
DCI[a]	All	3.56 (0.61–26.63)	4.49 (0.68–19.65)	4.38 (0–24.87)	5.49 (1.49–12.65)	7.65 (0	<0.05
	i.v.	1.73 (0.75–3.91)	4.54 (0.68–19.65)	3.79 (0.02–9.77)	4.12 (1.49–10.67)	5.00 (0.62	<0.05
	p.o.	6.49 (0.61–26.63)	4.34 (1.19–19.65)	7.96 (0–24.87)	6.13 (3.73–12.65)	11.92 (0	>0.05
		P**	0.05	>0.05	>0.05	>0.05	>0.05
CXI[b]							
	All	1.30 (0–28.44)	0.33 (0–9.34)	1.09 (0–14.38)	1.92 (0.09–9.39)	3.56 (0	>0.05
	i.v.	1.30 (0.04–3.9)	0.08 (0.01–3.32)	0.31 (0–3.24)	0.645 (0.09–4.66)	0.19 (0	>0.05
	p.o.	1.52 (0–28.44)	0.42 (0–9.34)	2.87 (0–14.38)	4.51 (0.61–9.39)	7.16 (0	>0.05
		P**	>0.05	>0.05	>0.05	>0.05	>0.05
IPM[c]							
	All	0 (0–17.73)	0 (0–4.12)	0 (0–81.64)	0 (0–4.91)	0 (0	>0.05
	i.v.	0 (0–1.47)	0 (0–0.04)	0 (0–4.93)	0 (0–4.91)	0 (0	>0.05
	p.o.	2.67 (0–17.31)	1.75 (0–4.12)	0 (0–81.64)	0 (0)	0 (0–59.33)	>0.05
		P**	<0.05	<0.05	>0.05	>0.05	>0.05
Total[d]							
	All	6.50 (1.18–45.72)	8.35 (0.73–28.99)	5.78 (2.20–81.54)	7.89 (9.58–22.09)	15.18 (0	<0.05
	i.v.	3.45 (1.18–7.8)	5.26 (0.73–18.82)	4.66 (2.22–15.79)	6.12 (1.58–22.09)	8.13 (0.92	<0.05
	p.o.	13.25 (1.85–45.32)	5.245 (1.61–28.99)	29.15 (2.2–81.64)	10.635 (4.34–22.04)	22.43 (0	<0.01**
		P**	<0.05	>0.05	>0.05	>0.05	>0.05

[a] DCI = dechlorethyl ifosfamide.
[b] CXI = carboxy ifosfamide.
[c] IPM = isophosphoramide mustard.
[d] Total = DCI+CXI+IPM.

* Friedman test
** Mann-Whitney test
*** Significant only if data from the first three days is counted

values did not reach statistical significance (see Table 1). In the i.v. group, dechloroethyl ifosfamide excretion increased over the 5-day period from a median of 1.73% (range, 0.73%–3.91%) on day 1 to that of 7.65% (range, 0–21.26%) on day 5 (P <0.05, Friedman test) (see Fig. 4). In the oral group, urinary excretion increased from 6.50% (range, 0.62%–26.63%) on day 1 to 11.92% (range, 0–13.3%) (median of only 5 cases; P >>0.05, Friedman test).

Excretion of carboxyifosfamide showed large interpatient variability, with a median value of 1.30% (range, 0–28.44%) for both groups on day 1 (see Fig. 5). There was no statistically significant difference in the median values between oral and i.v. administration; furthermore, there was no statistically significant increase in carboxyifosfamide levels over the 5-day period in either group.

Urinary levels of isophosphoramide mustard were very low, with a median value of 0 (range, 0–17.31%) on day 1 for both groups. Oral administration of ifosfamide resulted in statistically significantly higher levels of isophosphoramide mustard than did i.v. application on the first 2 days of drug administration (P <0.05, Mann-Whitney test). In both the i.v. and oral groups there was no statistically significant increase in isophosphoramide mustard excretion over the 5-day period (P >>0.05, Friedman test); however, certain individual patients did show a marked increase.

The median value for the urinary excretion of ifosfamide for both groups was 2.58% (range, 0–6.37%); there was no difference between the oral and i.v. groups (P >>0.05, Mann-Whitney test). Over the 5-day period of ifosfamide administration, there was no increase in urinary

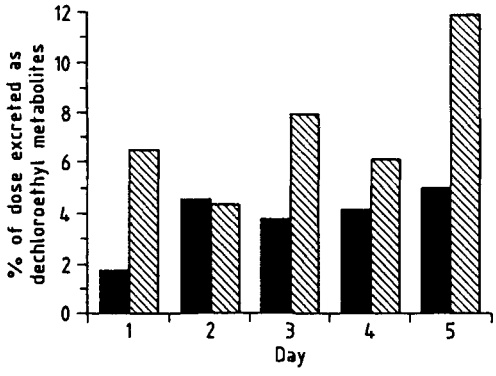

Fig. 4. Urinary excretion of dechloroethyl ifosfamide according to route of administration. ■, i.v.; □, p.o.

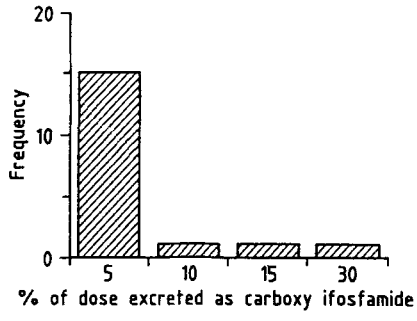

Fig. 5. Distribution of carboxyifosfamide excretion in patients receiving i.v. and oral ifosfamide

ifosfamide excretion (P >>0.1, Friedman test). The percentage of the delivered dose recovered as alkylating metabolites did not correlate with the observed CNS or haematological toxicity (P >>0.05, Kendall correlation coefficient). Furthermore, there was no correlation between any individual metabolite levels and the development of CNS or haematological toxicity (p >>0.05, Kendall correlation coefficient).

Discussion

This paper describes a relatively easy method for assaying urinary levels of ifosfamide, dechloroethyl ifosfamide, isophosphoramide mustard and carboxyifosfamide following parent-drug administration. Oral administration resulted in a higher recovery of alkylating metabolites (dechloroethyl ifosfamide, isophosphoramide mustard and carboxyifosfamide) from the urine, although this only reached statistical significance on the 1st day of ifosfamide administration. The failure to reach statistical significance on other days may in part be due to the fact that low numbers of patients in the oral arm received the full 5-day course of treatment due to the development of neurotoxicity, usually on day 3, necessitating early termination of treatment in these patients. Higher levels of metabolites following oral administration implies a significant first-pass metabolism of ifosfamide. This might explain the higher degree of CNS toxicity seen with this route of administration. Furthermore, it would suggest that a metabolite rather than the parent drug was responsible for the aetiology of the neurotoxic syndrome. However, some studies have reported an almost complete oral bioavailability [3, 18], a result at variance with that reported here. Moreover, our assessment of the effect of route of administration on ifosfamide metabolism is based on the determination of individual urinary metabolites rather than plasma AUC values and the assumption contained therein. Nevertheless, the small route difference observed suggests that the metabolite responsible for causing the neurotoxicity either is of low abundance (and thus very potent) or is one whose detection escapes our assay procedure.

From Table 1 it is apparent that only a small percentage of the dose was recovered from the urine as alkylating metabolites. Part of this may be due to the condensation of metabolites with mesna; however, this contrasts with the findings of Allen et al. [1], who recovered 41.2% of the radioactivity of [^{14}C]-labelled ifosfamide as urinary ifosfamide metabolites in patients receiving 1.6–2.4 g/m^2 i.v. ifosfamide. This discrepancy may be due to the formation of non-alkylating metabolites. Possible candidates are conjugates associated with glutathione, as it is well recognised that glutathione transferase is capable of detoxifying nitrogen mustards [4]. Furthermore, such conjugates would not react with NBP and hence could not be assayed using the methodology described in this paper. Therefore, it may be that although the total fraction of the ifosfamide dose metabolised was the same with the two routes, the fraction metabolised to alkylating metabolites may be greater than that metabolised to non-alkylating metabolites with the oral route, and the reverse situation may apply for i.v. administration. Evidence for differing profiles of metabolites following oral administration comes from Juma and colleagues [12], who demonstrated higher levels of alkylating activity in the serum of patients receiving oral cyclophosphamide than those observed when the same patients received an i.v. dose of the drug. These authors also demonstrated an oral bioavailability of nearly 100% for cyclophosphamide and concluded that a different profile of metabolites was produced by oral administration of the drug.

The major component of the urinary metabolites measured was dechloroethyl ifosfamide; the levels recovered were similar to those described by Norpoth [16]. This contrasts with cyclophosphamide metabolism, where the major urinary metabolite in man is carboxy cyclophosphamide [17]. The products of the N-dealkylation of ifosfamide are dechloroethyl ifosfamide and chloroacetaldehyde. Chloroacetaldehyde has recently been implicated in the neurotoxic syndrome [8]; it is chemically related to chloral hydrate, and the symptoms of ifosfamide-associated encephalopathy are similar to those seen in patients suffering from chloral hydrate toxicity. Chloroacetaldehyde itself has been shown to be neurotoxic in rats [13]. The present data demonstrate that urinary excretion of dechloroethyl ifosfamide (Table 1) is higher with oral administration, suggesting that chloroacetaldehyde levels would be expected to be higher with this route of administration. Disappointingly, urinary dechloroethyl ifosfamide levels failed to correlate with the development and severity of neurotoxicity seen in the patients studied.

This may be due to the small number of patients in this study; alternatively urinary levels of one metabolite may not accurately reflect plasma levels of another. Table 1 shows a high degree of inter-patient variability in the excretion of dechloroethyl ifosfamide; therefore, inter-patient variability in the *N*-dealkylation of ifosfamide may play a role in the development of neurotoxicity.

Urinary levels of carboxyifosfamide also showed great inter-patient variability, as can been seen from Fig. 5, which suggests that carboxyifosfamide excretion may be bimodal: 90% of patients excreted <8% of the delivered dose, whereas one patient excreted >28%. It is known that aldehyde dehydrogenase, the enzyme responsible for the formation of carboxyifosfamide, exhibits genetic polymorphism [7]. Most of the present work relates to ethanol metabolism, but oxazaphosphorine-resistant tumour cells have been shown to have elevated levels of aldehyde dehydrogenase [11]. Treatment of these cell lines with aldehyde dehydrogenase inhibitors such as disulfiram restores their sensitivity to that of wild-type cells. In addition, organs that are high in aldehyde dehydrogenase levels, such as the liver and kidney, are capable of deactivating cyclophosphamide in vitro, and these organs do not show toxicity when the drug is given in vivo [6]. Therefore, such a genetic polymorphism may exist in patients and, hence, phenotypic differences in aldehyde dehydrogenase activity may be a determinant of efficacy/toxicity in patients receiving oxazaphosphorines. Patients with high aldehyde dehydrogenase activity might be expected to show therapeutic failure, whereas those with low aldehyde dehydrogenase activity might develop drug sensitivity and/or toxicity. Recently, in a small series of patients receiving cyclophosphamide, 24-h urine analysis by TLC-PD revealed a deficiency (0.1%–0.3% of the dose) in carboxycyclophosphamide excretion [10]; carboxycyclophosphamide was hitherto considered to be the major metabolite of cyclophosphamide.

The levels of isophosphoramide mustard recovered from the urine were far lower than the urinary phosphoramide mustard excretion previously seen following cyclophosphamide administration (approximately 18% [5, 17]). This is probably a reflection of *N*-dealkylation of ifosfamide, reducing the availability of parent drug for 4-hydroxylation and subsequent isophosphoramide mustard production, the ratio of plasma parent drug to the 4-hydroxy metabolite being greater for ifosfamide than for cyclophosphamide [20].

Urinary excretion of dechloroethyl ifosfamide increased over the 5-day period. It is interesting to note that when encephalopathy occurred, it generally did so around or after the 3rd day of drug administration, presumably the time at which chloroacetaldehyde levels were maximal. In addition, large increases in isophosphoramide mustard excretion were seen in some patients, which suggests auto-induction of ifosfamide metabolism. Various authors have shown that the elimination half-life falls and the total body clearance increases on repeated daily administration of ifosfamide, and our data would tend to support this hypothesis [14, 18, 19].

In conclusion, the present study shows that oral administration of ifosfamide is associated with a higher degree of CNS toxicity and results in higher urinary levels of alkylating metabolites, especially dechloroethyl ifosfamide. Metabolic patterns were highly variable between patients, which may in part be due to a polymorphism in carboxyifosfamide production, analogous to that recently reported for cyclophosphamide metabolism. However, there are major differences in the metabolism of the two drugs; ifosfamide can undergo significant *N*-dealkylation, which may account for the occurrence of CNS toxicity that is occasionally seen with ifosfamide administration but never with cyclophosphamide. We therefore suggest that oral administration produces higher levels of chloroacetaldehyde, the culprit responsible for this toxicity. There is also some evidence to suggest that daily repeated administration of the drug causes auto-induction of ifosfamide metabolism.

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