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Determination of urinary 2- and 3-dechloroethylated metabolites of ifosfamide by high-performance liquid chromatography

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ABSTRACT

In vivo oxidation of chloroethyl side-chains on ifosfamide produces the toxin chloroacetaldehyde. Production of this labile metabolite can be indirectly quantitated by monitoring the excretion of the residual 2- and 3-dechloroethylated ifosfamide. Urinary ifosfamide and the two dechloroethylated metabolites were extracted into chloroform from alkalinized salt-saturated urine, followed by high-performance liquid chromatographic separation using an acetonitrile gradient on a reversed-phase column and ultraviolet detection at 190 nm. In five patients given 1.6 g/m^2 ifosfamide, 11–30% of the dose was excreted over 24 h as unchanged drug, 11–21% as 3-dechloroethylated and 3–10% as 2-dechloroethylated ifosfamide.

INTRODUCTION

Administration of ifosfamide to patients with cancer can produce neurotoxic, nephrotoxic, and bladder toxic effects that are attributable in part to the metabolite chloroacetaldehyde [1–3]. Quantitation of urinary chloroacetaldehyde does not reflect the total production of this metabolite because of its lability and non-renal clearance; however, the proportion of ifosfamide metabolized to chloroacetaldehyde can be judged indirectly by monitoring the excretion of the residual 2- and 3-dechloroethylated ifosfamide (2-DCE, 3-DCE) (Fig. 1).

Concentrations of 2-DCE, 3-DCE, and ifosfamide have been determined in urine samples by ^{31}P nuclear magnetic resonance spectroscopy (NMR) [4,5], but the signals for 3-DCE and ifosfamide overlap. In one study, these signals were resolved by extraction of the urine with chloroform and ethyl acetate followed by reconstitution and reanalysis in ethanol [4]. Thin-layer chromatography (TLC) has been used to separate urinary 2-DCE and 3-DCE from ifosfamide and other metabolites [6–8], but the two dechloroethylated compounds comigrate and their summed concentration is less than that reported by NMR. Urinary 2-DCE has been quantitated by capillary gas chromatography [8], using a procedure that

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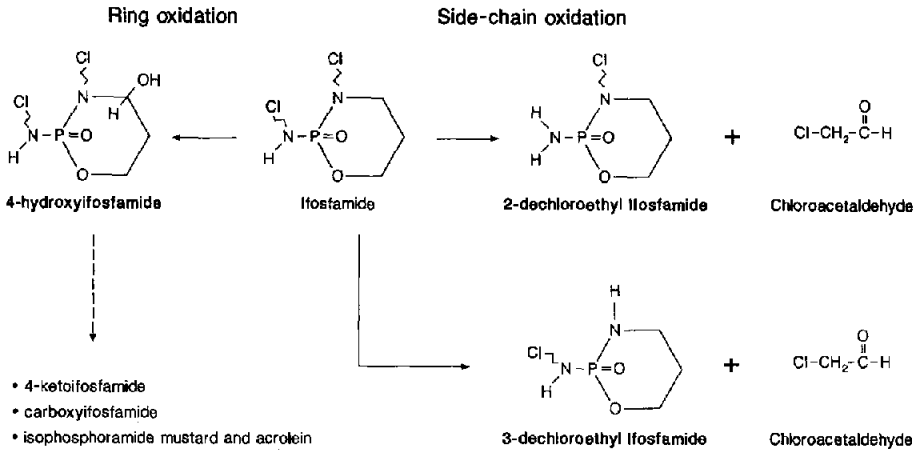

Fig. 1. Principal metabolites of ifosfamide.

separates the enantiomers of 2-DCE, 3-DCE, and ifosfamide without derivatization [9].

Ifosfamide has been analyzed by high-performance liquid chromatography (HPLC) and UV detection after liquid–liquid [10] and solid-phase extraction [11] from blood, but conditions for the separation and quantitation of 2-DCE and 3-DCE have not been reported. We anticipated that their detection should be possible because as much as 30% of ifosfamide has been reported to be metabolized to 2-DCE and 3-DCE [4,5]. We compared the efficiency of extraction of 2-DCE, 3-DCE, and ifosfamide by several techniques and identified chromatographic conditions to separate and quantitate 2-DCE and 3-DCE in human urine.

EXPERIMENTAL

Chemicals

Ifosfamide, 2-DCE, 3-DCE, carboxyifosfamide, and ketoifosfamide were gifts from Asta Pharma (Bielefeld, Germany). The structural identity of the 2-DCE and 3-DCE was confirmed by mass spectrometry (Dr. Robert F. Struck, Southern Research Institute, Birmingham, AL, USA); we evaluated their purity by TLC followed by reaction with 4-(4-nitrobenzyl)pyridine. Other reagents were HPLC grade.

Patient treatment and urine collection

We studied five newly diagnosed and previously untreated patients who were given 1.6 g/m^2 ifosfamide intravenously over 15 min. At 0, 4 and 6 h after the end of the ifosfamide infusion, each patient also received three 15-min infusions of

400 mg/m^2 mesna, a free sulfhydryl agent that prevents bladder cystitis by neutralizing toxic ifosfamide metabolites.

Just before administration of ifosfamide, patients were asked to void their bladders and a 20-ml aliquot of urine was set aside to be used as the matrix for the standard curve. Urine was then collected for 24 h at 6-h intervals after ifosfamide injection, and 7-ml aliquots were frozen at −70°C.

Calibration and controls

Standard curves were prepared by addition of pure analytes from a stock solution to the urine obtained from the patient before therapy. The stock solution of 50 m*M* ifosfamide, 50 m*M* 2-DCE, and 50 m*M* 3-DCE was prepared in acetonitrile and stored at −70°C. The pretherapy urine was thawed, centrifuged at 1300 *g* for 10 min to remove amorphous salts, and diluted with physiologic saline to 300 mg/l creatinine. The stock standard solution was then diluted in this urine to obtain 0, 25, 50, 100, 250, 500, and 1000 μM concentrations of the three analytes. These urine standards were processed in parallel with samples and analyzed by linear regression to calibrate the assay.

To control for inter-assay variation, 100 and 1000 μM concentrations of the analytes were prepared in urine from a healthy donor, and aliquots were frozen.

Sample preparation

Urine aliquots were thawed and centrifuged at 1300 *g* for 10 min to remove amorphous salts. Creatinine concentrations were determined and specimens were diluted with physiologic saline to 300 mg/l creatinine so as to be similar to the standards prepared in pretherapy urine. The diluted urine samples and standards were made alkaline by addition of 0.2 ml of 1 *M* sodium hydroxide followed by addition of an excess of crystalline sodium chloride. Analytes from the salt-saturated and alkalinized urine were extracted into 8.5 ml of chloroform by vortex-mixing and then rocking the samples for 10 min. After centrifugation at 1300 *g* for 10 min, the lower layer was filtered through phase separator paper (Whatman International, Maidstone, UK) into 10-ml glass tubes and the chloroform evaporated over approximately 1 h in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY, USA). The residue was reconstituted in 300 μl of 10% acetonitrile and filtered through 0.2-μm Nylon-66 membranes (Microfilterfuge tubes, Rainin Instrument, Woburn, MA, USA) in a microcentrifuge at 14 000 *g* for 1 min. Sample volumes of 50 μl were injected for analysis.

Other sample extraction procedures that were tested included a solid-phase extraction with a C_{18} column [12] and extraction into acetonitrile with salt [13].

Instrumentation

Analyses were performed using Beckman System Gold software configured with a Model 406 analog-digital converter, two Model 110 B pumps, a Model 407 autosampler, and a Model 166 UV detector set to 190 nm (Beckman, Houston,

TX, USA). Analytes were separated on a Waters μBondapak C_{18} (10 μm, 30 cm × 3.9 mm I.D.) column and Waters Guard-Pak precolumn containing the same packing material (Waters, Milford, MA, USA). Separations were also attempted with a Waters μBondapak phenyl column.

Chromatography

A 20-min step gradient was used to separate the analytes and equilibrate the column. The mobile phases were 10% and 60% acetonitrile in water (v/v). The flow-rate was 2 ml/min. The 2-DCE and 3-DCE were eluted using 10% acetonitrile for 3.5 min. The proportion of the 60% acetonitrile mobile phase was then increased from 0 to 30% over a 0.5-min interval, thereby increasing the acetonitrile concentration of the mixed mobile phase to 28%. After 8.5 min at 28% acetonitrile (during which ifosfamide eluted), the proportion of the 60% acetonitrile mobile phase was decreased to 0% over 0.5 min and the column was equilibrated with 10% acetonitrile for 7 min before the next sample injection.

RESULTS AND DISCUSSION

Reproducibility and limit of detection

The coefficients of variation for 2-DCE, 3-DCE, and ifosfamide in pooled urine on nine days were 9.5, 4.2, and 7.0% at a level of 100 μ*M* and 4.8, 5.7, and 8.4% at 1000 μ*M*. These values were similar to those for HPLC assays of ifosfamide alone reported by Margison *et al.* [10] (5.1% for a 77 μ*M* control over six assays) and Burton and James [11] (4.8% for a 100 μ*M* control sample over six assays).

The limit of detection varied between patients because of differences in urine composition. Metabolite concentrations as low as 2–5 μ*M* were detected in some specimens, but concentrations below 25 μ*M* were infrequently observed in aliquots collected during the 24-h collection period.

Calibration

The calibration curves were linear to concentrations as high as the 1000 μ*M* standard. The mean correlation coefficients for twelve assays on different days for 2-DCE (0.9987 ± 0.0014 S.D.), 3-DCE (0.9987 ± 0.0010), and ifosfamide (0.9983 ± 0.0015) exceeded 0.998. The slopes of the calibration curves for 2-DCE (mean ± S.D. 168 ± 23; range 145–232), 3-DCE (73 ± 10; 64–99), and ifosfamide (278 ± 33; 240–341) varied between runs because the standards were diluted in pretherapy urines from different patients; changes in the column and in detector response may have also contributed. The mean slopes of the calibration curves for 2-DCE, 3-DCE, and ifosfamide differed because of the lower proportions of metabolites extracted from urine and their lower relative absorbances at equimolar concentrations. In aqueous solutions, the absorbances of the chromatographic peaks for 2-DCE and 3-DCE at concentrations ranging from 100 to

1000 μM were 89 and 38% of that for ifosfamide at 190 nm using the Model 166 detector. No improvement in the signal-to-noise ratio was obtained at 195 to 205 nm.

Recovery

A simple procedure involving liquid–liquid extraction into chloroform sufficed for our purpose. Using the procedure described by Margison *et al.* [10] for the determination of ifosfamide, we recovered 48, 34, and 100% of 1 m*M* 2-DCE, 3-DCE, and ifosfamide added to urine as compared to aqueous standards. By increasing the sample volume to 3 ml, reducing the volume of 1 *M* sodium hydroxide to 0.2 ml, adding an excess of sodium chloride to the urine, and reducing the volume of chloroform to 8.5 ml, we increased the extraction of 2-DCE and 3-DCE.

In one experiment, at urinary concentrations ranging from 100 to 1000 μM, the ranges of 2-DCE and 3-DCE extracted were 67–83 and 72–83%, respectively. Similar results (2-DCE 69%, 3-DCE 70%) were obtained by analysis of the calibration curve data, noting that the slopes for 2-DCE and 3-DCE were lower than those for ifosfamide by an amount proportional to the relative efficiency of extraction and relative absorbance. The means of the ratios of the slopes ($n = 12$ pairs) for 2-DCE to ifosfamide and 3-DCE to ifosfamide were 62 and 27%, respectively. These values were 69 and 70% of the respective relative absorbances of 89 and 38% for equimolar aqueous solutions described above.

The following modifications of the liquid–liquid extraction procedure were of no additional benefit. Duplicate and triplicate extractions yielded negligible improvements in recovery. Chloroform extraction of the amorphous salts and cellular debris removed by centrifugation of urine yielded no detectable 2-DCE or 3-DCE, and less than 4% of the ifosfamide present in the supernatant. At neutral pH, the recovery of 3-DCE but not 2-DCE was diminished by about half. Acidification of the urine by substitution of 1 *M* hydrochloric acid for the sodium hydroxide reduced the extraction into chloroform of both 2-DCE and 3-DCE to 1%, probably due to hydrolysis of the nitrogen–phosphorus bond.

Solid-phase extraction by use of a C_{18} column as described by Hardy *et al.* [12] yielded 91% recovery for ifosfamide, but only 36 and 41% for 2-DCE and 3-DCE. Extraction into "salted-out" acetonitrile as described by Rustum and Hoffman [13] recovered 96% of the ifosfamide, but only 38 and 30% of the 2-DCE and 3-DCE, and there was a large interfering solvent front due to acetonitrile. Acidification of the urine reduced the recovery of the metabolites to less than 1%.

Chromatography

Fig. 2 shows a typical chromatogram for the separation of 2-DCE, 3-DCE, and ifosfamide in a urine from a patient given ifosfamide. The step gradient from 10 to 28% acetonitrile rapidly eluted the three peaks. Isocratic conditions were tested, but the elution time for ifosfamide at acetonitrile concentrations of 5- 15%

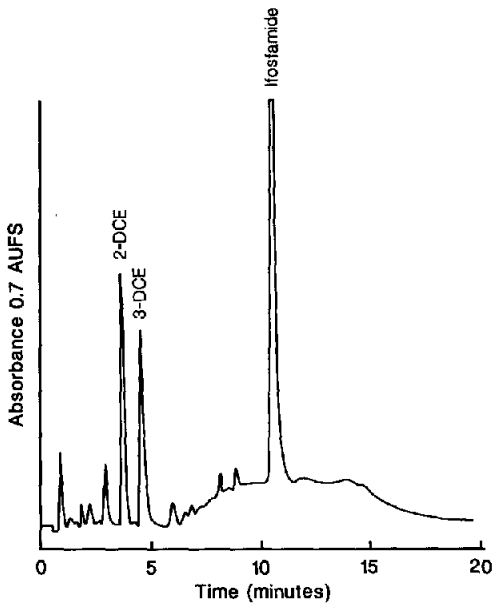

Fig. 2. Chromatogram of a urine sample obtained between 0 to 6 h after administration of 1.6 g/m^2 ifosfamide. The measured concentrations of 2-DCE, 3-DCE, and ifosfamide were 330, 805, and 615 μM, respectively.

was excessive, and at acetonitrile concentrations above 20% the peaks for 2-DCE and 3-DCE were not resolved. Attempts to resolve 2-DCE and 3-DCE using the μBondapak phenyl column were not successful at acetonitrile concentrations ranging from 10 to 30%, pH 3.0–7.5, and 5–50 mM phosphate buffer.

The chromatographic peak for high concentrations of ifosfamide overlapped with a later eluting background peak that was present in some patients. The interfering peak was present in the pre- and post-therapy urines, producing nearly identical areas provided that the urines were diluted to 300 mg/l creatinine. The peaks were resolved at lower concentrations of ifosfamide. At higher ifosfamide concentrations, the two peaks overlapped, but the area of the interfering peak was in the worst case less than 5% of that for ifosfamide.

Drugs given to patients concurrently with ifosfamide that did not produce interfering peaks included: acetaminophen, diphenhydramine, etoposide, hydroxyzine pamoate, ibuprofen, lorazepam, medroxyprogesterone acetate, meperidine, metuclopramide, morphine, norethindrone, phenytoin, promazine, promethazine, ranitidine, sulfamethoxazole, thiethylperazine, and trimethoprim.

Other chromatographic peaks occasionally present in post- but not in pre-ifosfamide urine specimens might represent other metabolites of ifosfamide or other drugs given concurrently with ifosfamide. These peaks had the following retention times: 4.3 min (between 2-DCE and 3-DCE), 5.6 min (after 3-DCE), 8.5

min (on the slope of the gradient), 9.8 min (probably ketoifosfamide and carboxyifosfamide), and 10.6 min (before the ifosfamide peak).

Aqueous solutions containing pure ketoifosfamide and carboxyifosfamide coeluted at 9.9 min, but were extracted from alkalinized and salt-saturated urine into chloroform with efficiencies of only 2 and 1%, respectively.

Solvents that could not be used at high concentration because of absorbance at 190 nm included ethyl acetate, acetic acid, and acetonitrile.

Patient data

Table I summarizes the data for the urinary excretion of ifosfamide, 2-DCE, and 3-DCE in the five patients given 1.6 g/m^2 ifosfamide. The measured values for the 6-h aliquots have been summed to obtain the total amount of unchanged ifosfamide and metabolites for the 24-h period. The proportion of the dose excreted as 2-DCE (3–10%) and 3-DCE (11–21%) over 24 h is similar to that reported for three patients (2-DCE, 4.5–11.6%; 3-DCE, 15.2–21.3%) by NMR [5]. By contrast, values obtained by quantitation of the single band for 2-DCE and 3-DCE on TLC plates have been lower, depending in part on the technique used to visualize the band. Using densitometry of plates sprayed with 5-pyridinealdehyde-2-benzothiazolylhydrazone, Norpoth [6] showed data for three patients given 5–6 g of ifosfamide over 2 h from which the summed recovery of 2-DCE and 3-DCE can be calculated to range from 6 to 20% over 24 h. Densitometry of photographs of the reaction of the metabolites with 4-(4-nitrobenzyl)-pyridine yielded even lower values [7]. In ten adults given 1.5 g/m^2 ifosfamide, only 0.75–3.91% of the dose was excreted as the sum of 2-DCE and 3-DCE during the first 24 h, as compared to 15–31% in our five children after a similar dosage; also, the range for the urinary excretion of ifosfamide was reported to be 0–6.37% as compared to 11–30% in our children.

The ratio of the sum of 2-DCE and 3-DCE to ifosfamide ranged from 0.7 to 2.8 (Table I), demonstrating inter-patient variation in side-chain oxidation as suggested qualitatively by TLC studies [6,7]. The median ratio of 1.2 was similar to that obtained by NMR [4] in a single patient given 2 g/m^2 ifosfamide and studied over eight days; the median ratio of DCE to ifosfamide can be calculated from the NMR data shown to be 1.27.

This simple HPLC procedure separates and reproducibly quantitates ifosfamide and its two dechloroethylated metabolites in urine, avoiding problems with the resolution of 2-DCE and 3-DCE and with the sensitivity of detection and reproducibility of quantitation inherent with TLC and NMR. In the five patients studied, 15–31% of the dose of ifosfamide administered was recovered within 24 h in the urine as 2-DCE and 3-DCE. It can be inferred that the patient was exposed to an equimolar amount of the toxin chloroacetaldehyde (mean 2025 μmol, Table I); however, none of these children developed neurotoxicity after their initial exposure to this relatively low dosage of ifosfamide. This HPLC procedure should prove useful to evaluate the potential contribution of chloro-

TABLE I

EXCRETION AND RECOVERY OF A 1.6 g/m² DOSE OVER 24 h OF UNCHANGED IFOSFAMIDE AND 2- AND 3-DECHLOROETHYLATED METABOLITES IN FIVE PREVIOUSLY UNTREATED PATIENTS WITH MALIGNANT SOLID TUMORS

Values in parentheses represent recovery.

Patient No.	Dose given (μmol)	Excreted (μmol)				Ratio of DCE/IFOS	Total DCE + IFOS (μmol)
		Ifosfamide	2-DCE	3-DCE	Total DCE		
1	6498	712 (11%)	186 (3%)	783 (12%)	969 (15%)	1.4	1700 (26%)
2	9073	1496 (16%)	537 (6%)	1039 (11%)	1576 (17%)	1.1	3071 (34%)
3	9625	1071 (11%)	954 (10%)	2029 (21%)	2983 (31%)	2.8	4055 (42%)
4	8766	2008 (23%)	776 (9%)	1726 (20%)	2502 (29%)	1.2	4510 (51%)
5	9502	2821 (30%)	584 (6%)	1513 (16%)	2097 (22%)	0.7	4918 (52%)
Mean	8693	1622 (18%)	607 (7%)	1418 (16%)	2025 (23%)	1.4	3651 (41%)

acetaldehyde towards the risk of neurotoxicity and nephrotoxicity by monitoring dechloroethylation in dosage escalation studies of ifosfamide, by comparing the metabolism of ifosfamide administered by different schedules, *e.g.*, continuous infusion and bolus injection, and by evaluating the effect on ifosfamide metabolism of earlier or concurrent therapy with other drugs, *e.g.*, in patients treated with large cumulative dosages of the nephrotoxic anticancer drug cisplatin [14].

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