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Combined thin-layer chromatography–photography–densitometry for the quantification of ifosfamide and its principal metabolites in urine, cerebrospinal fluid and plasma

Alan V. Boddy* and Jeffrey R. Idle

Pharmacogenetics Research Unit, Department of Pharmacological Sciences, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH (UK)

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ABSTRACT

A method has been devised for the determination of the anticancer drug ifosfamide and its principal metabolites in urine, plasma and cerebrospinal fluid (CSF). The urine and CSF samples are adsorbed onto Amberlite XAD-2 eluting the compounds of interest with methanol. Plasma is deproteinated using cold acetonitrile and centrifuged to yield a clear supernatant. The eluate and supernatant are analyzed by thin-layer chromatography, with spot visualization using 4-(4-nitrobenzyl)pyridine. The plates are photographed for subsequent densitometric analysis. The intra-assay coefficient of variation for each compound in both urine and plasma was less than 10% and the lower limit of detection was 1 μg/ml. The method provides a means of determining the full spectrum of metabolic products of ifosfamide in patients and will allow detailed investigations of variability in metabolism and pharmacokinetics of this drug.

INTRODUCTION

Ifosfamide is a DNA-alkylating agent, a structural isomer of cyclophosphamide used in the treatment of adult and pediatric tumours [1,2]. The drug requires metabolic activation via a number of intermediates to exert its cytotoxic effect [3]. Because of the multiple pathways of metabolism of this drug (Fig. 1), the disposition of the active and inactive metabolites has been studied intensively using a variety of techniques [4–9]. To date, however, no single method has been described for the simultaneous quantitation of the parent drug and each of the major metabolites (2- and 3-dechloroethylifosfamide, 4-ketoifosfamide and isophosphoramide mustard). These metabolites represent a wide variety of chemical species and have no suitable chromophore for UV detection, thus precluding conventional gas and liquid chromatographic techniques. A common method of analysis is to estimate total alkylating activity using the Epstein colorimetric assay [10], involving alkylation of 4-(4-nitrobenzyl)pyridine (NBP) and exposure to alkaline conditions. A method has been developed combining thin-layer chromatographic (TLC) separation of the metabolites with the NBP visualization reaction, followed by rapid photography of the labile blue spots and finally densitometric analysis of a full-size print of the TLC plate. This method is similar to one described previously for the metabolites of cyclophosphamide in urine [11]. The chromatographic conditions have been modified for ifosfamide and the assay used to quantify levels of drug and metabolites in urine, plasma and cerebrospinal fluid (CSF).

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EXPERIMENTAL

Materials

Chemical structures of the authentic compounds analysed are given in Fig. 1. Chemical nomenclature used is that cited in Chemical Abstracts together with the widely used trivial nomenclature of the metabolites. The following substances were the gift of Asta-Werke (Frankfurt, Germany) and were authenticated in their laboratories: N,N′-bis(2-chloroethyl)phosphorodiamidic acid (isophosphoramide mustard, IPM), 3-[(N,N′-bis(2-chloroethylamino)phosphinyloxy]propanoic acid (carboxyifosfamide, CXIFO), 2-(2-chloroethyl)aminotetrahydro-2-oxide-2H-1,3,2-oxazaphosphorine (3-dechloroethylifosfamide, DCCP), 3-(2-chloroethyl)-aminotetrahydro-2-oxide-2H-1,3,2-oxazaphosphorine (2-dechloroethylifosfamide, DCIFO), 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-2-oxide-4H-1,3,2-oxazaphosphorine-4-one (4-ketoifosfamide, 4-KetoIFO). N,N′-Bis-(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine 2-oxide (ifosfamide, IFO) was obtained as Mitoxana for intravenous use from ASTA Medica (Cambridge, UK).

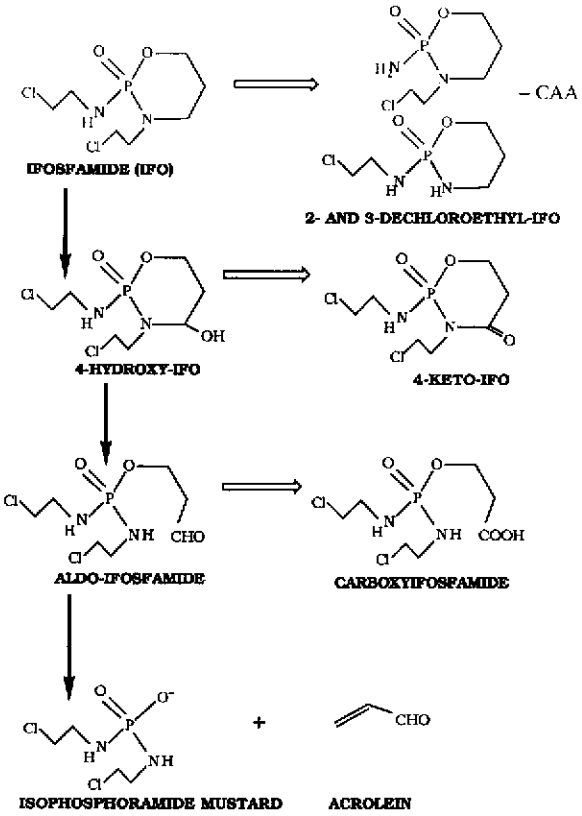

Fig. 1. Metabolic activation and inactivation reactions of ifosfamide.

Inorganic reagents were of AnalaR grade and organic solvents were HPLC grade. NBP and cyclophosphamide (CP) were obtained from Sigma (Poole, UK).

Preparation of samples for TLC

Urine and CSF. Urines from patients treated with ifosfamide or blank human urine spiked with authentic standards (2–50 μg ml^{-1}) were treated as follows. Each urine sample (1 ml) was applied to an XAD-2 Spe-Ed solid-phase extraction cartridge (500 mg/3 ml, Laboratory Impex, Teddington, UK) on a vacuum manifold and 50 μl of internal standard (cyclophosphamide, 500 μg/ml in methanol) was added to each. The cartridge was washed with 3 ml of water and then left with air passing through it for 2–3 h until dry. Drug and metabolites were eluted with three 1-ml volumes of methanol. The methanol was evaporated to dryness at 40°C under vacuum.

Plasma. Plasma (750 μl) was added to 750 μl of cold acetonitrile and 50 μl of internal standard in a 1.5-ml Eppendorf tube. This was then vortex-mixed and spun in a microfuge (Heraeus, Osterode, Germany) for 15 min. The clear supernatant was transferred to another Eppendorf tube (with a punctured lid) and evaporated to dryness at 40°C under vacuum.

TLC procedures

Dry residues from the above procedures were reconstituted in methanol (70 μl) and 30 μl were applied to 20 cm × 10 cm TLC plates [precoated high-performance thin-layer chromatographic (HPTLC) glass-backed silica gel 60; E. Merck, Darmstadt, Germany] which had been pre-eluted with methanol and dried at 150°C for 10 min. An automated, "Linomat IV", TLC sample applicator was used (Camag, Muttenz, Switzerland). Chromatography was performed in glass TLC tanks, saturated with solvent. The mobile phase

was dichloromethane–methanol–glacial acetic acid (90:8:1, v/v/v), which was allowed to rise to a height of at least 9.5 cm. After drying, the plates were run again in a second mobile phase of chloroform–methanol–glacial acetic acid (90:60:1, v/v/v) to a height of 2 cm. The plates were dried again and sprayed for at least 10 s with 5% NBP in acetone–0.2 *M* acetate buffer, pH 4.6 (8:2, v/v), dried and then resprayed for 10 s. Plates were heated in an oven at 150°C for 10 min and left to cool.

Photographic procedure

Plates were dipped in 3% methanolic potassium hydroxide to reveal the blue spots formed from alkylated NBP. The plates were photographed within 10 s of dipping because of the unstable nature of the chromophore [10,11]. The photographic equipment consisted of a Polaroid MP3 Land camera, the film plane of which was positioned 65 cm above the base on which the plates were placed. The plates were illuminated at a 45° angle by two tungsten halogen 150-W lamps. The film used was 3.25 × 4.25 in. Polaroid 655, which produces both an instant positive image and a self-developing negative. The lens was a Polaroid 105 mm at f22 with a half second exposure. To ensure uniform exposure and printing, a Kodak standard grey scale was photographed with each plate.

The negative was enlarged to the exact size of the original plate using a 135 mm lens at f11 for 5 s. Ilford Multigrade glossy paper was used, developing in Ilfospeed developer for 1 min. Uniform exposure was ensured by comparison of the Kodak grey scale with the original.

Densitometric analysis

The photographs of the plates were mounted on the platform of a Camag Scanner II densitometer and scanned in the reflection/absorption mode at 500 nm using a beam, 5 mm long by 4 mm wide. The program CATS 3 (Camag) was used to integrate the areas under the chromatogram peaks. The peak areas for ifosfamide and metabolites were divided by the area under the internal standard (CP) peak and the peak-area ratio used for calibration. Each plate contained samples and at least six tracks derived from spiked urine, plasma or phosphate buffer (pH 7.4) for CSF samples containing known concentrations of authentic standards (2–50 μg/ml). Calibration curves were obtained for ifosfamide and each of the metabolites and used to determine the concentrations in patient urine and plasma samples.

RESULTS

With the chromatographic conditions described above, all of the IFO metabolites and the internal standard (CP) were well separated (Fig. 2). Separation of IPM from the polar residues of the biological fluids was achieved by running the plate in a second mobile phase for 2 cm. Photography of the plates gave a permanent record of the density of the labile blue spots which could then be scanned to yield a chromatogram. A photograph of one track from a urine sample, together with the corresponding chromatogram, is shown in Fig. 3. Calibrations were linear over the range 2–50 μg/ml and details of the calibration lines, recoveries and variability of the assay applied to urine are give in Table I. Replicate analysis of samples on different plates gave coeffcients of variation (C.V.) less than 15%. Comparable results were obtained in plasma and CSF (buffer). Recovery was over 75% for all the compounds and the C.V. in peak-area ratio on one plate did not exceed 9%. The slope of the calibration varied over a ten-fold range for the different metabolites, reflecting differences in ability to alkylate NBP, in the absorbance of the conjugate formed and in the shape of the spots.

Blank urine, CSF and plasma did not interfere with the assay. Only other alkylating agents which would also react with NBP could potentially produce an interfering spot on the chromatogram. However, combinations of IFO with other alkylating agents are not in routine clinical use.

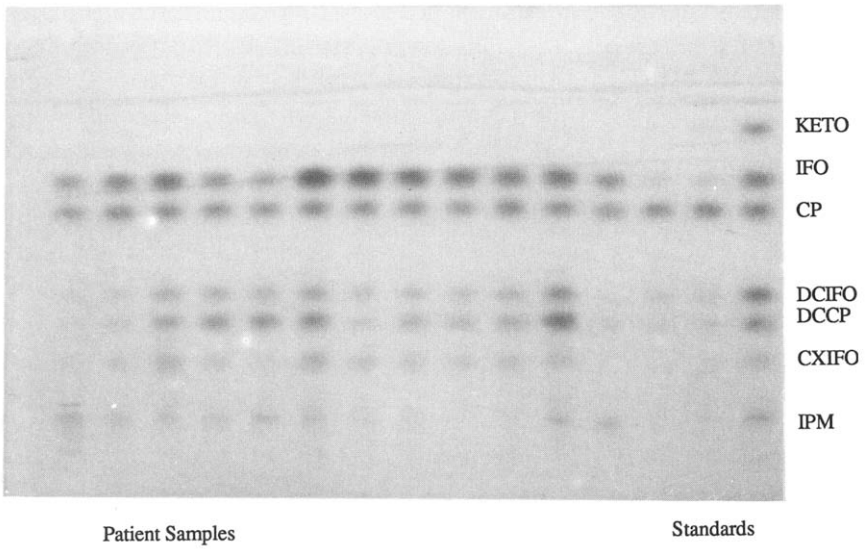

Fig. 2. Photograph of section of TLC plate and Kodak grey scale. The first twelve tracks are diluted samples of patient urine and the last three are authentic standards (5, 5 and 25 μg/ml) derived from spiked, blank urine.

Pattern of IFO metabolite excretion in human urine

The chromatography of IFO and its metabolites in urine is illustrated in Fig. 2. Fig. 4 shows the cumulative excretion of IFO and its metabolites in the urine of one patient receiving a 24-h infusion of 5 g/m^2 ifosfamide. The measurement of the parent drug and metabolites in sequential urine samples allows calculation of recovery of each compound as a percentage of the dose administered. A phenotypic deficiency in the excretion of carboxy metabolite of cyclophosphamide has been previously reported [12], but the same deficiency was not observed in patients receiving ifosfamide [13]. These studies involved relatively small numbers of patients and the present assay will be used to study a larger population, including pediatric subjects.

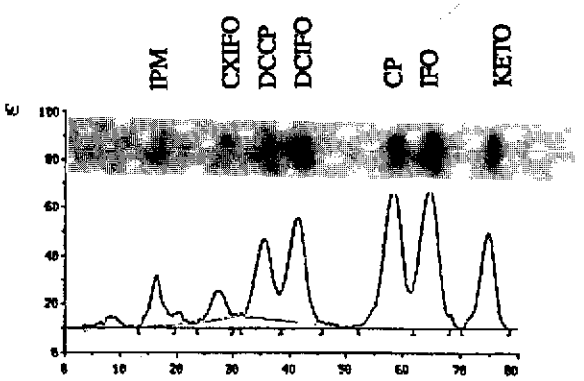

Fig. 3. Photograph of one track from a developed TLC plate and the corresponding chromatogram. The peaks shown are for IPM, CXIFO, DCCP, DCIFO, CP (internal standard), IFO and KetoIFO at a concentration of 25 μg/ml.

The rate of excretion of drug and metabolites, together with plasma data, permit calculation of renal clearance values for the drug and metabolites during the steady-state period. The renal clearance can in turn be related to the renal function of the patient.

Plasma concentration–time profile of IFO and its metabolites in plasma

Fig. 5 shows the plasma concentration of IFO and four metabolites at different times during and after a 24-h infusion of 5 g/m^2 ifosfamide. Although small quantities of the keto metabolite could be seen in urine, none was seen in plasma.

It can be seen that parent drug and metabolites attain steady state in 24 h and that they decline in parallel after the infusion is stopped. The area under the plasma concentration–time curve (AUC) can be used to determine the exposure of the patient to the drug and to the individual me-

TABLE I

CHROMATOGRAPHIC AND ASSAY PARAMETERS FOR IFOSFAMIDE AND ITS METABOLITES

Compound	Recovery[a] (%)	R_F[b]	r^2[c]	Slope	Variation[d] (one plate)	Variation[d] (between plates)
IFO	84	0.70	1.000	0.0303	4.1	10.5
IPM	73	0.09	0.998	0.00446	8.3	12.8
CXIFO	75	0.26	0.991	0.0148	2.6	13.0
DCCP	89	0.34	0.999	0.0441	3.9	8.6
DCIFO	91	0.42	0.999	0.0297	1.1	8.9
KetoIFO	76	0.86	0.997	0.0163	2.2	15.3
CP (I.S.)	88	0.59			3.0	8.7

[a] Recovery relative to methanolic solution applied directly to the plate.

[b] R_F value for IPM is after second chromatographic step.

[c] r^2 values and slopes are for typical chromatograms from the same plate using standards in urine. Comparable results were obtained in plasma and CSF (buffer).

[d] Variations are given as the coefficient of variation (%) at a concentration in urine of 5 μg/ml, except the internal standard.

tabolites. The degree of dechloroethylation is important as each mole of dechloroethylated metabolite is associated with the formation of an equimolar quantity of chloroacetaldehyde. This compound has been associated with the clinical toxicity of IFO. Similarly, the formation of IPM involves the release of an equimolar quantity of acrolein, another toxic metabolite. The relative amounts of IPM and CXIFO formed reflect the balance of activation and inactivation reactions in the body as a whole.

It is important to determine the pharmacokinetics of the drug and metabolites in plasma and urine since measurement in either body fluid alone would yield less information about the formation and elimination kinetics of each metabolite and about the ultimate fate of the administered dose. Determination of drug and metabolite concentrations in CSF will allow an assessment to be made of the penetration of IFO and its active metabolites into the brain.

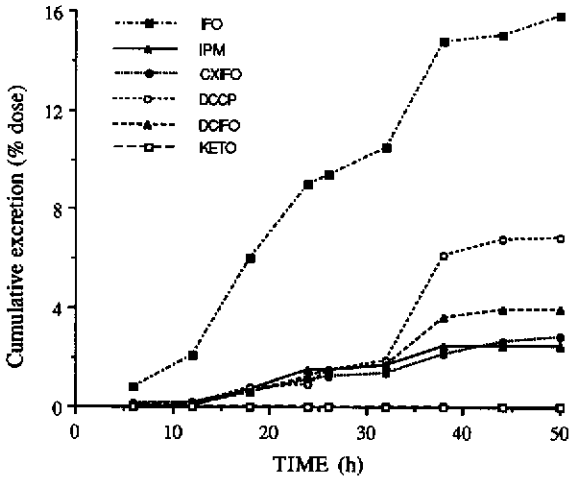

Fig. 4. Cumulative excretion of ifosfamide and its metabolites in urine, during and 24 h after a 24-h infusion of 5 g/m² ifosfamide in an adult patient.

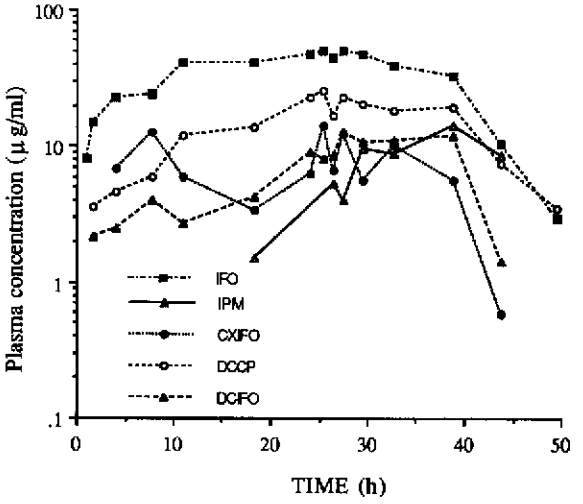

Fig. 5. Plasma concentrations of ifosfamide and its metabolites during and 24 h after a 24-h infusion of 5 g/m² ifosfamide in an adult patient.

DISCUSSION

That the therapeutic and toxic effects of the oxazaphosphorines are mediated by their metabolites has been well documented [2]. Variations in the toxicity and tumour responsiveness to oxazaphosphorines have been associated with deficiency or overactivity of one or more metabolic pathways [8,14–16]. Thus, in order to understand the significance of the different routes of metabolism, it is necessary to use a sensitive, selective assay capable of determining the major metabolites in both urine and plasma. TLC with subsequent photography and densitometry permits such measurements to be made. It is a relatively simple and cheap assay method which does not require the use of radiolabelled drug, complex derivatizations or expensive gas or high-performance liquid chromatographic equipment.

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