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Analysis of ifosfamide, 4-hydroxyifosfamide, N2-dechloroethylifosfamide, N3-dechloroethylifosfamide and iphosphoramide mustard in plasma by gas chromatography–mass spectrometry

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Abstract

A sensitive and specific method for the simultaneous quantitation of ifosfamide (IF), 4-hydroxylifosfamide (4-OHIF), N2-dechloroethylifosfamide (N2D), N3-dechloroethylifosfamide (N3D) and iphosphoramide mustard (IPM) has been developed using gas chromatography–mass spectrometry (GC–MS) with an ion-trap mass spectrometer. Deuterium labeled analogues for each of these analytes were synthesized as the internal standards. The labile 4-OHIF in plasma was first converted to the more stable cyanohydrin adducts before dichloromethane extraction. IPM was extracted by C_{18} reversed-phase resin. All analytes were converted to their silyl derivatives before GC–MS analysis. The sensitivity limits ranged from 0.1 to 0.5 μg/ml when 100 μl of plasma was used. This method was validated with within-run coefficients of variation less than 5% ($n = 8$) and between-run coefficients of variation less than 12% ($n = 6$). The method was applied to the determination of plasma levels of IF and metabolites in the rat.

1. Introduction

Ifosfamide [IF, 3-(2-chloroethyl)-2-(2-chloroethylamino) - 2H - tetrahydro - 1,3,2 - oxazaphosphorin-2-oxide], a structural isomer of cyclophosphamide (CP), is widely used in cancer chemotherapy either as a single agent or as a component in combination regimens [1–3]. The drug itself is inactive, requiring hepatic microsomal transformation to exert its pharmacological and toxicological effects. The most important metabolic pathway is 4-hydroxylation generating 4-hydroxyifosfamide (4-OHIF), which may readily partition across cells [4]. 4-OHIF is generally thought to equilibrate with its open ring tautomer, aldoifosfamide (aldoIF). β-Elimination of acrolein from AldoIF generates iphosphoramide mustard (IPM), which has been considered as the ultimate intracellular alkylating metabolite [5,6]. Dealkylation of the 2-chloroethyl chains generates N2- and N3-dechloroethylifosfamide (N2D and N3D), with the concomitant production of chloroacetyl aldehyde

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which has been implicated in the neurotoxicity observed in patients receiving IF therapy [7,8].

Several analytical techniques have been applied to quantitate IF, N2D and N3D, including GC [9–26], HPLC [27–31], TLC [7,32,33], total alkylating activity assay using N-nitrobenzylpyridine (NBP) [34], total radioactivity assay after administration of ^{3}H- or ^{14}C-labeled IF [35], and GC–MS [36,37]. A recently reported ^{31}P-NMR method [38,39] allows simultaneous quantitation of all phosphorus-containing metabolites in patient urine. However, its intrinsic sensitivity is low, hampering its application to pharmacokinetic studies. Few efforts have been devoted for the analysis of 4-OHIF due to its chemical instability. In fact, no direct and specific method is available in the literature. Instead, an indirect fluorometric method [23,40,41] using 4-aminophenol has been commonly used to measure this metabolite in plasma and urine based on the assay of 4-OHIF-derived acrolein. It is assumed that the acrolein of metabolic origin from IF in plasma prior to deproteinization is negligible, since acrolein, once formed, should quickly react with surrounding macromolecules. Thus, plasma levels of acrolein generated from degradation of 4-OHIF following deproteinization were thought to represent the true levels of 4-OHIF. This assumption, however, has not been substantiated as yet and, in fact, considerable doubts exist, since acrolein can also be generated from secondary metabolites of IF such as 4-hydroxy-N2D and 4-hydroxy-N3D [42]. In spite of various analytical methods developed, few complete pharmacokinetic profiles of IF and its metabolites have been reported [23,43]. This may attribute to potential problems in applying these methods in biological system.

Thus, a composite GC–MS has been developed for the analysis of IF and its major metabolites, using stable isotope analogues for each analyte as the internal standard and is

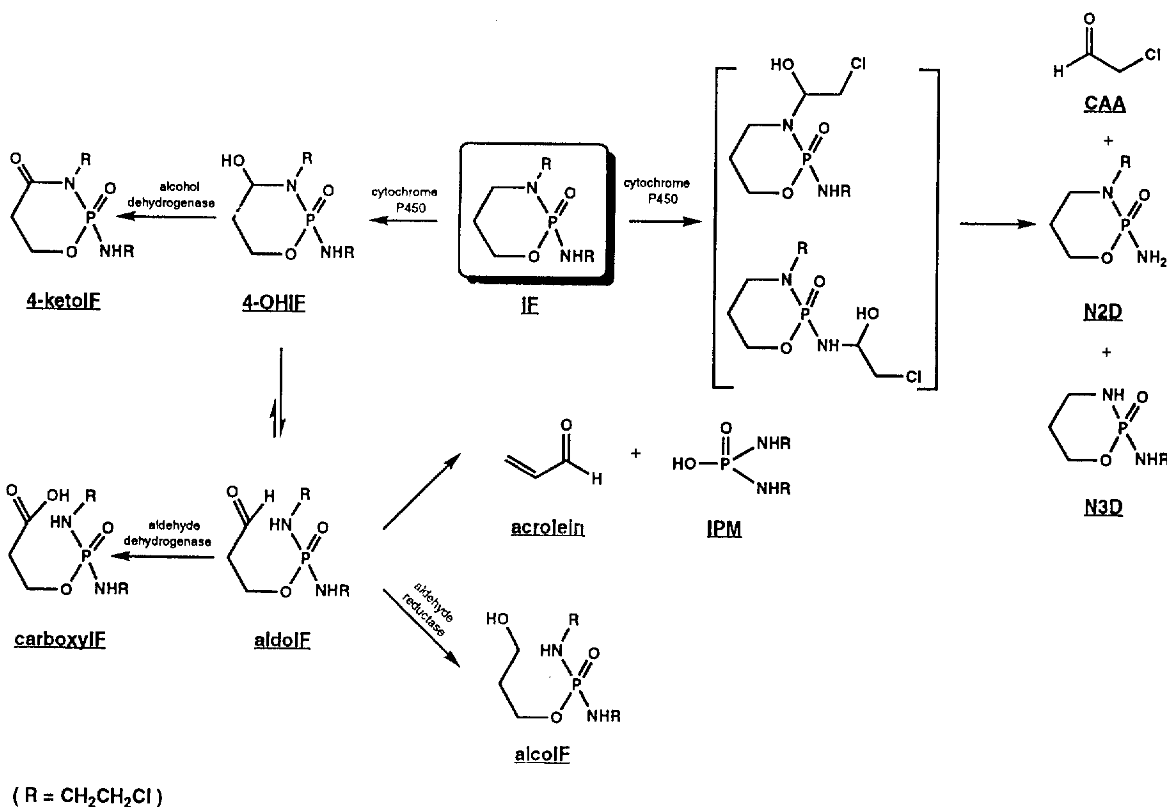

Fig. 1. Metabolic pathways of ifosfamide.

described here. 4-OHIF was isolated from rat plasma and analyzed as the cyanohydrin adduct. Total IPM was determined from a modified method developed in this laboratory [6] and true IPM level can be obtained from subtraction of 4-OHIF level from total IPM level. Using this method, plasma levels of IF and its metabolites including 4-OHIF, N2D, N3D and IPM in the rat following i.v. administration of IF were determined.

2. Experimental

2.1. Chemicals

IF and IPM were provided by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, the National Cancer Institute. 4-OHIF, N2D and N3D were synthesized in this laboratory (Wang and Chan, unpublished data). The internal standard 6,6,2′,2′,2″,2″-hexadeuterio-4-hydroxyifosfamide (4-OHIF-d_6) was prepared by the reduction of 6,6,2′,2′,2″,2″-hexadeuterio-4-hydroperoxyifosfamide (4-OOHIF-d_6) with sodium thiosulfate immediately before use [44]. All synthetic reagents were obtained from Aldrich (Minneapolis, MN, USA) and all HPLC grade organic solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and N-trimethylsilylimidazole (TMSI) were purchased from Pierce (Rockford, IL, USA). C_{18} reversed-phase resin was obtained from Analytichem International (Harbor City, CA, USA).

2.2. Cyanohydrin adduct of 4-OHIF/aldoIF in plasma

Rat plasma sample or rat blank plasma (100 μl) spiked with IF, 4-OHIF, N2D, N3D was placed in a culture tube (16×100 mm) containing 100 μl of 1.5 *M*, pH 8 KCN solution and 1 μg of IF-d_6, 3 μg of 4-OHIF-d_6, and 200 ng each of N2D-d_4 and N3D-d_8. The tube was vortex-mixed and placed at room temperature for 30 min.

2.3. Extraction and derivatization

IF, cyanohydrin adduct of 4-OHIF/aldoIF, N2D, N3D, and their internal standards in the plasma sample were extracted with 5 ml of dichloromethane. The mixture was shaken for 15 min in a horizontal shaker (Eberbath, Ann Arbor, MI, USA). The organic phase was separated after centrifugation, and evaporated under a stream of nitrogen. The residue was derivatized with 35 μl of MSTFA for 60 min at 120°C and an aliquot (0.5 μl) of the derivatized sample was analyzed by GC–MS.

A procedure developed in this laboratory was modified for the analysis of total IPM [6]. Rat plasma sample or rat blank plasma (100 μl) spiked with IPM was placed in a culture tube containing 1 μg of IPM-d_8 as the internal standard, and the mixture was loaded onto a disposable Poly-prep column (Bio-Rad, Richmond, CA, USA) containing 400 mg of C_{18} reversed-phase resin. The column was washed with 0.5 ml of ice-cold saline followed by centrifugation at 200 *g* for 20 min to remove water. The column was then eluted with 1 ml of methanol, and the methanol fraction was collected and evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was derivatized with 35 μl of a mixture of BSTFA and TMSI (5:1, v/v) at 120°C for 60 min and an aliquot (0.5 μl) of the derivatized sample was analyzed by GC–MS.

2.4. Gas chromatography–mass spectrometry

A Finnigan ITS40 Ion Trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) directly coupled to a Model 3300/3400 Varian gas chromatograph (Walnut Creek, CA, USA) with a capillary splitless injector was used for the analysis. The temperatures of the injection port, transfer line, and source were maintained at 220, 260 and 230°C, respectively. The analysis was carried out under chemical ionization condition with ammonia as the reagent gas and the emission current was set at 10 μA. Helium was used as the carrier gas with a head pressure of 15 p.s.i. (ca. 10^5 Pa). A DB-5 fused-silica capillary col-

umn (30 m × 0.25 mm I.D.) coated with a 0.25-μm thickness film of methylsilicone plus 5% phenyl methylsilicone (J&W Scientific, Folsom, CA, USA) was used for the separation.

The temperature of the oven was programmed at 150°C for 2 min and increased to 190°C at a rate of 5°C/min, and then to 250°C at a rate of 15°C/min. The final temperature was held for 3 additional min for a total of 17 min.

2.5. Assay validation

The within-run precision of assay was evaluated at concentrations of 5 μg/ml each for IF, 4-OHIF and IPM and 1 μg/ml each for N2D and N3D in eight replicates. Between-run precision was evaluated in six different occasions and the slopes from six standard curves were used to calculate the coefficient of variation.

2.6. Recovery evaluation

The recovery of IF, N2D, N3D, and IPM were estimated by comparing the ratios of the extracted analytes to the respective unextracted internal standards with those of the unextracted pairs. For 4-OHIF, since the extraction was conducted following its conversion to the cyanohydrin adduct, the recovery was conducted on the derivative. Comparison of the ratio of the extracted cyanohydrin adduct of 4-OHIF from plasma to its unextracted lyophilized adduct of the internal standard with the same of the unextracted lyophilized adduct pair gave the recovery of the 4-OHIF adduct.

The concentrations of analytes used for recovery evaluation were the same as those for assay validation.

2.7. Animal studies

The jugular vein of a male Sprague–Dawley rat (Harlan, Indianapolis, IN, USA) weighing 285 g was cannulated under ethyl ether anesthesia [45]. After 2 hour recovery, IF dissolved in normal saline (1 ml) at 40 mg/kg was injected into the animal through the cannula. The cannula was washed three times with 0.3 ml each of 0.9% sodium chloride solution. Blood samples (0.2–0.6 ml) were collected in heparinized tubes at 0, 5, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420 min, after dosing. Plasma was obtained from each sample by centrifugation at 2000 *g* and 4°C for 2 min. The plasma was obtained and immediately stored at −70° until analysis.

2.8. Data analysis

Regression analysis and pharmacokinetic model fitting were accomplished using a RSTRIP Program (MicroMath Scientific Software, Salt Lake City, UT, USA) on an IBM PC.

2.9. Synthesis of the internal standards

4,4,5,5,6,6-hexadeuterio-ifosfamide (IF-d_6)

(a) Ethyl 2,2-dideuteriocyanoacetate. A mixture of ethyl cyanoacetate (10.0 g) and deuterium oxide (10.0 g) was heated to reflux for 15 min. The reaction mixture was extracted with CH_2Cl_2 (50 ml × 2). The extract was dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated in vacuo to give a colorless oil (9.2 g). The same procedure was repeated 4 times to effect complete isotope exchange. The final recovery was 7.3 g (73.0%). NMR (C^2HCl_3) d 1.33 (t, $J = 7.1$ Hz, 3H, $-CH_3$); 4.29 (q, $J = 7.1$ Hz, 2H, $-CH_2-$).

(b) 1-Amino-1,1,2,2,3,3-hexadeuteriopropan-3-ol. to a cooled (−78°C) suspension of lithium aluminum deuteride (2.0 g) in anhydrous THF (30 ml) was added dropwise a solution of ethyl 2,2-dideuterio-cyanoacetate (3.45 g) in THF (10 ml). The mixture was stirred for 1 h at ambient temperature and the temperature was then raised to reflux. The reaction mixture was stirred for 10 additional h. A saturated solution of sodium sulfate (5 ml) was added slowly to the cooled (0°C) mixture to quench the reaction. The solid material was collected by filtration and extracted with THF (200 ml) using a Soxhlet apparatus for 2 days. The filtrate and the extract were combined and concentrated to give a yellow oil (1.79

g, 73.6%). NMR (C^2HCl_3) d 1.65 (bs, 3H, OH, NH_2).

(c) 2-Chloro-4,4,5,5,6,6-hexadeuteriotetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide.
Into a cooled (−78°C) solution of phosphorus oxychloride (2.21 g, 14.4 mmol) in anhydrous CH_2Cl_2 (20 ml) was added slowly a mixture of 1-amino-1,1,2,2,3,3-hexadeuteriopropan-3ol (1.17 g, 14.4 mmol) and triethylamine (2.92 g, 28.8 mmol) in CH_2Cl_2 (5 ml). The reaction mixture was stirred for 1 h at room temperature. Anhydrous ethyl ether (50 ml) was added to precipitate triethylamine hydrochloride. The solid was removed by filtration and the filtrate was evaporated to give the product as a yellow oil, which was used for the next stage without further purification.

(d) 2-(2-Chloroethyl)-amino-4,4,5,5,6,6-hexadeuteriotetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide (N3D-d_6). To a CH_2Cl_2 solution (20 ml) of the oxazaphosphorine chloride was added 2-chloroethylamine hydrochloride (1.68 g, 14.4 mmol). Triethylamine (2.92 g, 28.8 mmol) in CH_2Cl_5 (5 ml) was then added dropwise to the suspension. The reaction mixture was stirred for 2 h at room temperature. The solvent was removed by rotary evaporation in vacuo and the residue extracted with hot acetone (20 ml × 4). Evaporation of the solvent in the extract afforded a semi-solid (2.52 g), which was chromatographed on a silica gel column using CH_2Cl_2–EtOH (15:1) as the eluent to give N3D-d_6 (0.58 g, 23.1% from the amino alcohol): mp 105–107°C; R_F 0.18 (CH_2Cl_2–acetone, 1:3). The chemical identity of N3D-d_6 was confirmed by GC–MS analysis.

(e) 2-(2-Chloroethyl)-amino-3-chloroacetyl-4,4,5,5,6,6-hexadeuteriotetrahydro-2H,1,3,2-oxazaphosphorin-2-oxide (N3-chloroacetyl N3D-d_6). To a cooled (0°C) solution of N3D-d_6 (0.48 g, 2.35 mmol) in THF (20 ml) was added a solution of chloroacetyl chloride (0.79 g, 7.1 mmol, 3:1 excess) in THF (5 ml). The progress of reaction was monitored by TLC. Once the acylation was completed, the volume of the solution was reduced in vacuo to 10 ml. Water (10 ml) was then added and the mixture extracted with CH_2Cl_2 (50 ml × 4). The CH_2Cl_2 extract was dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated to give a colorless oil (0.92 g). Column purification (silica gel) with CH_2Cl_2–acetone (4:1) as the eluent gave the product as a colorless oil (0.56 g, 85.0%): R_F 0.72 (CH_2Cl_2–acetone, 1:1).

(f) IF-d_6. Into a cooled (−78°C) solution of 1 *M* BH_3 in anhydrous THF (12 ml, 6:1 excess) was added dropwise N3-chloroacetyl N3D-d_6 (0.56 g, 2.00 mmol) in THF (10 ml). The reaction mixture was stirred for 1 h at ambient temperature. TLC showed complete disappearance of the starting material. Water (10 ml) was then added to destroy the remaining BH_3. After the removal of THF in vacuo, the resulting mixture was extracted with CH_2Cl_2 (20 ml × 4). The organic phase was dried over anhydrous sodium sulfate. After filtration, the solvent in the filtrate was evaporated to give a colorless oil (1.00 g). Column chromatography (silica gel) using CH_2Cl_2–acetone–methanol (40:3:1) as the eluent gave IF-d_6 as a colorless oil (0.29 g, 54.7%): R_F 0.33 (CH_2Cl_2–acetone, 1:1), which crystallized during storage in a freezer (−76°C). The chemical identity was confirmed by GC–MS analysis comparing with authentic IF. Under the GC–MS conditions described previously, this product gave a major peak at *m/e* 231 (one Cl), the ratio 231/230/229 indicated the product to contain IF-d_6 and the d_5 and d_4 analogues in the ratio of 70.4:27.5:2.2.

6,6,2′,2′,2″,2″-Hexadeuterio-4-hydroperoxyifosfamide (4-OOHIF-d_6)

(*a*) 1,1-*Dideuterio*-3-*buten*-1-*ol*. To a cooled (−78°C) suspension of lithium aluminum deuteride (1.16 g, 27.6 mmol) in anhydrous THF (30 ml) was added slowly a solution of vinyl acetic acid (4.74 g, 55.1 mmol) in THF (10 ml). The mixture was heated to reflux for 5 h. Water (3 ml) was added dropwise to destroy the remaining deuteride while the reaction flask was immersed in an ice-bath. The solid material was

collected by suction filtration, and was extracted with THF for two days using a Sohxlet extractor. The filtrate and the extract were combined and dried over anhydrous sodium sulfate. Removal of THF gave 1,1-dideuterio-3-buten-1-ol as a light brown oil (2.05 g, 50.2%): R_F 0.84 (CH_2Cl_2–acetone, 1:1); NMR (C^2HCl_3) d 1.55 (bs, 1H, OH) 2.31 (d, J = 6.7 Hz, C2-H), 5.08–5.20 (m, 2H, C4-H), 5.70–5.88 (m, 1H, C3-H).

(*b*) *O-*(1,1-*Dideuterio-*3-*butenyl*)-*N,N′-bis*(2-*chloro-*2,2-*dideuterioethyl*)*phosphorodiamidate.* To a stirred solution of phosphorus oxychloride (1.04 g, 6.76 mmol) in CH_2Cl_2 (20 ml) was added dropwise a solution of 1,1-dideuterio-3-buten-1-ol (0.5 g, 6.76 mmol) and triethylamine (0.68 g, 6.76 mmol) in CH_2Cl_2 (5 ml) at −78°C. The reaction mixture was stirred for 1.5 h. 2-Chloro-2,2-dideuterioethylamine hydrochloride (1.60 g, 13.5 mmol) was added to the reaction mixture, followed by triethylamine (2.73 g, 27.0 mmol) in CH_2Cl_2 (5 ml). The reaction mixture was stirred for 2 additional hours at room temperature. Precipitated triethylamine hydrochloride was removed by filtration. The filtrate was washed with distilled water (10 ml × 3) and the organic phase was dried over anhydrous sodium sulfate. After filtration, the solvent in the filtrate was evaporated to give a yellow oil (1.48 g, 75.9%). Column purification (silica gel) with CH_2Cl_2–acetone (5:1) as the eluent gave the product (0.63 g, 32.3%) as a colorless oil: R_F 0.41 (CH_2–Cl_2-acetone, 1:1); NMR (C^2HCl_3) d 2.42 (d, J = 6.7 Hz, 2H), 2.94–3.08 (m, 2H, 2 × NH), 3.18–3.33 (m, 4H, 2 × NH-*CH*2-), 5.08–5.20 (m,2H, -CH=CH_2), 5.72–5.90 (m, 1H, -*CH*=CH_2).

(c) 4-OHIF-d_6. A stirred solution of the phosphorodiamidate (0.43 g, 1.53 mmol) in aqueous acetone (1:1, 30 ml) was bubbled with O_3 at a flow-rate of 3.5 ml/min (90v, 90w) at 0°C for 1 h. Hydrogen peroxide (30%, 0.5 ml) was then added to the ozonized solution. After standing at 4°C for 3 days, acetone in the reaction mixture was removed in vacuo and the aqueous residue was extracted with CH_2Cl_2 (50 ml × 4). The combined extract was dried over anhydrous sodium sulfate. After filtration, the filtrate was evaporated in vacuo to give a colorless oil (0.3 g), which crystallized by addition of acetone (0.3 ml) and ethyl ether (3 ml). After standing at −70°C overnight, the crystals were collected by filtration and washed with cooled (0°C) ethyl ether to give 4-OOHIF-d_6 as a white solid (0.08 g). The mother liquor was concentrated and the resulting oil was re-dissolved in aqueous acetone (1:1, 30 ml). Hydrogen peroxide (30%, 0.5 ml) was added to the mixture. After standing at 4°C for 2 days, acetone was removed in vacuo and the remaining aqueous solution was extracted with CH_2Cl_2 (50 ml × 5) and the second crop of 4-OOHIF-d_6 (0.07 g) was obtained by a similar treatment. The remaining mother liquor was treated similarly and the third crop (0.01 g) was obtained. The overall yield was 35.0%. R_F 0.74 (CH_2Cl_2–acetone, 3:1). The chemical identity was confirmed by GC–MS analysis after the conversion to the cyanohydrin adduct. From the relative intensities of m/z 418/417, the ratio between 4-OOHIF-d_6 and its d_5 and d_4 analogues was found to be 97.1:2.8:0.1.

1′,1′,2′,2′-Tetradeuterio-N2-Dechloroethylifosfamide (N2D-d_4)

(a) Methyl N-1,1,2,2-tetradeuterio-2-hydroxylethyl-3-amino propionate. To a solution of methyl acrylate (0.66 g, 7.69 mmol) in THF (10 ml) was added 1,1,2,2-tetradeuterioethanolamine (0.50, g, 7.69 mmol). The mixture was stirred at room temperature for 2 days. The solvent was removed in vacuo to give a colorless oil (1.16 g, 100%): R_F 0.07 (CH_2Cl_2–acetone, 1:1). The product was used for the subsequent step without purification.

(b) Methyl N-2-chloro-1,1,2,2-tetradeuterioethyl-3-amino propionate. Into a cooled (0°C) solution of methyl N-1,1,2,2-tetradeuterio-2-hydroxylethyl-3-amino propionate (1.16 g, 7.68 mmol) in CH_2Cl_2 (10 ml) was added dropwise thionyl chloride (1.82 g, 15.4 mmol). The mixture was heated gradually to 60°C. The progress of chlorination was followed by TLC. Once the reaction was completed, water (20 ml) was added to destroy the remaining thionyl chloride. The mixture was extracted with CH_2Cl_2 (20 ml × 4) and

the extract was discarded. The aqueous solution was neutralized to pH 9–10 using saturated NaOH solution. The resultant mixture was extracted with CH_2Cl_2 (20 ml × 4). The combined organic phase was washed with water (10 ml) and dried over anhydrous sodium sulfate. After filtration, the solvent in the filtrate was removed in vacuo to give the product as a pale yellow oil (1.20 g, 92.2%): R_F 0.57 (CH_2Cl_2–acetone 1:1).

(c) N-2-Chloro-1,1,2,2-tetradeuterioethyl-3-aminopropan-1-ol. To a cooled (−78°C) suspension of lithium aluminum deuteride (0.27 g, 7.1 mmol) in THF (20 ml) was added dropwise a solution of methyl N-2-chloro-1,1,2,2-tetradeuterioethyl-3-amino propionate (1.20 g, 7.1 mmol) in THF (5 ml). The mixture was stirred at room temperature for 1 h. Water (1 ml) in THF (10 ml) was added to destroy the remaining hydride. After filtration, the solid material was extracted with CH_2Cl_2. The filtrate and extract were combined and dried over anhydrous sodium sulfate. After filtration, the volume of the filtrate was reduced in vacuo to about 10 ml. The resulting solution was used directly for the subsequent step.

(d) 2-Chloro-3-(2-chloro-1,1,2,2-tetradeuterioethyl)-tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide. To a cooled (−78°C) solution of phosphorus oxychloride (1.08 g, 7.08 mmol) in CH_2Cl_2 (10 ml) was added the solution of N-2-chloro-1,1,2,2-tetradeuterioethyl-3-aminopropan-1-ol obtained from the previous step. Triethylamine (1.43 g, 14.2 mmol) in CH_2Cl_2 (5 ml) was then added to the mixture. The reaction mixture was stirred for 1 h at room temperature. The solvent was removed in vacuo and the residue extracted with anhydrous ethyl ether. The extract was dried over anhydrous sodium sulfate. After filtration, the solvent in the filtrate was removed to give a colorless oil (1.23 g), which was purified by silica gel column chromatography using ethyl ether as the eluent.

(e) N2D-d_4. To the ethyl ether solution of the oxazaphosphorine chloride directly obtained from the previous step was bubbled with anhydrous ammonia at room temperature. After 3 h, when no more starting material could be detected by TLC, acetone (200 ml) was added to the mixture. Precipitated ammonium chloride was removed by filtration. The filtrate was dried over anhydrous sodium sulfate. After filtration, the solvent in the filtrate was removed to give colorless crystals (0.28 g, 19.6% from amine ester): mp 101–103°C; R_F 0.22 (CH_2Cl_2–acetone, 1:3). The chemical identity of the product was confirmed GC–MS analysis. The ratio m/z 239/238 showed N2D-d_4 and its d_3 and d_2 analogs to be present in the ratio of 97.9:2.0:0.1.

4,4,5,5,6,6,2′,2′-Octadeuterio-N3-dechloroethyl-ifosfamide (N3D-d_8)

(a) 2-Chloro-4,4,5,5,6,6-hexadeuterio-tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide. To a cooled (−78°C) solution of phosphorus oxychloride (1.97 g, 12.8 mmol) in anhydrous CH_2Cl_2 (40 ml) was added slowly a mixture of 1-amino-1,1,2,2,3,3-hexadeuteriopropan-3-ol (1.04 g, 12.84 mmol) and triethylamine (2.59 g, 25.7 mmol) in CH_2Cl_2 (20 ml). The reaction mixture was stirred for 4 h at room temperature. Anhydrous ethyl ether (50 ml) was then added to precipitate triethylamine hydrochloride. The soluble material was collected by filtration and concentrated to give the product as a yellow oil (1.48 g), which was used for the subsequent reaction without isolation.

(b) N3D-d_8. To a cooled (0°C) solution of the oxazaphosphorine chloride (1.48 g) in anhydrous CH_2Cl_2 (40 ml) was added previously synthesized 2-chloro-2,2-dideuterioethylamine hydrochloride (1.51 g, 12.8 mmol). Triethylamine (2.59 g, 25.7 mmol) in CH_2Cl_2 (10 ml) was then added dropwise to the suspension. The reaction mixture was stirred for 2 h at room temperature. The solvent was removed by rotary evaporation in vacuo. The residue was extracted with hot acetone (20 ml × 4). Concentration of the extract afforded a semi-solid (2.94 g). Silica gel column purification using CH_2Cl_2–EtOH (15:1) as the eluent gave N3D-d_8 as colorless crystals (0.59 g, 22.3% from amino alcohol): mp 107–109°C; R_F 0.18 (CH_2Cl_2–acetone, 1:3). The chemical identity of N3D-d_8 was confirmed by GC–MS analysis.

The ratio m/z 243/242/241 indicated that the product contained N3D-d_8 and the d_7 and d_6 analogues in the ratio of 88.0:11.1:0.9.

1′,1′,2′,2′,1″,1″,2″,2″-Octadeuterioiphosphoramide mustard (IPM-d_8)

(*a*) *2 - Chloro - 1,1,2,2 - tetradeuterioethylamine hydrochloride*. To a cooled (−78°C) 1 *M* HCl ethereal solution (20 ml) was added dropwise a solution of 1,1,2,2-tetradeuterioethanol amine (1.0 g, 15.38 mmol) in anhydrous ethyl ether (5 ml) while vigorous stirring was maintained. The amine alcohol hydrochloride salt was obtained as a white powder when the remaining HCl and ethyl ether were removed by rotary evaporation in vacuo. The salt was suspended in 1,2-dichloroethane (10 ml). Into the cooled (0°C) suspension was added thionyl chloride (5.49 g, 46.1 mmol). The reaction mixture was stirred for 30 min at room temperature, and was then heated gradually to 50–60°C. The progress of the chlorination was followed by TLC. The precipitated product was collected by filtration and washed with CH_2Cl_2 and acetone. Crystallization in ethyl alcohol gave the product as colorless crystals (1.76 g, 95.3%): mp 136–138°C (143–146°C for the unlabeled compound); R_F 0.58 (acetone–methanol, 1:1), MS m/z 84 (MH^+ of the free amine).

(*b*) *Phenyl N,N′-bis-(2-chloro-1,1,2,2-tetradeuterioethyl)diamidophosphate*. To a cooled (0°C) solution of phenyl dichlorophosphate (0.88 g, 4.17 mmol) in CH_2Cl_2 (15 ml) was added 2-chloro-1,1,2,2-tetradeuterioethylamine hydrochloride (1.0 g, 8.34 mmol). Triethylamine (1.68 g, 16.7 mmol) in CH_2Cl_2 (10 ml) was added dropwise into the stirred suspension. The reaction mixture was stirred at room temperature for 1 h. Acetone (200 ml) was then added to the mixture and precipitated triethylamine chloride was removed by filtration. The solvent in the filtrate was removed in vacuo to give a light yellow oil (1.68 g). The crude material was purified by silica gel column chromatography eluted with CH_2Cl_2–acetone (5:1) to give the product as a colorless oil (1.28 g, 100%): R_F 0.65 (CH_2Cl_2–acetone, 1:1); NMR (C^2HCl_3) d 3.07–3.48 (bs, 2H, 2 × NH), 7.12–7.40 (m, 5H, Ph-H).

(*c*) *IPM-d_8*. The phenyl diamidophosphate (1.28 g) was dissolved in CH_2Cl_2 (50 ml) and, after addition of 10% Pd/C (0.5 g), was hydrogenated at room temperature under ambient pressure. The progress of the hydrogenation was followed by TLC. When the starting material disappeared (4.5 h), the product along with the catalyst was collected by filtration and washed with CH_2Cl_2. The product was dissolved in methanol and the catalyst was then removed by filtration. Removal of the solvent in the filtrate in vacuo afforded crystalline IPM-d_8 (0.93 g, 96.9%): mp 114–115°C. GC–MS analysis confirmed its chemical identity and indicated the product contained IPM-d_8 and its d_7 and d_6 analogues in the ratio of 98.6:1.3:0.1.

Results

3.1. GC–MS analysis

The entire assay procedure is summarized in the flow chart (Fig. 2). As shown, IF and four metabolites can be analyzed in a single plasma sample which is divided into two portions for different extraction methods. One aliquot was processed for solvent extraction for IF, 4-OHIF/aldoIF, N2D and N3D and their respective deuterium labeled internal standards following stabilization of 4-OHIF/aldoIF by cyanohydrin formation. The other aliquot was processed by solid-phase extraction for IPM. During the solid-phase extraction step, however, 4-OHIF/aldoIF underwent complete degradation to form acrolein and IPM. Thus, the IPM measurement following solid-phase extraction represents the composite 4-OHIF/aldoIF and IPM (total IPM). For this reason, the true IPM levels were computed by subtraction of 4-OHIF/aldoIF from the total IPM on the molar basis.

Ion chromatograms are shown in Fig. 3A–I and Fig. 4A–C. As shown, no interference in ion regions of interest in the blank rat plasma was found. During the silylation procedure, IF and

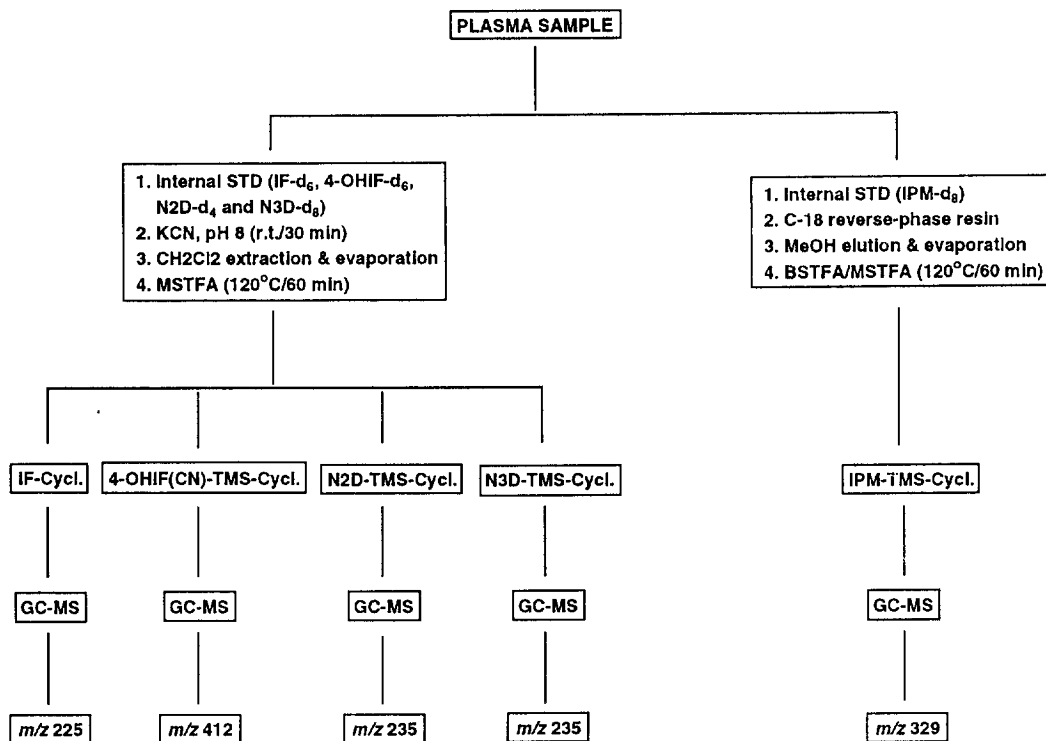

Fig. 2. Comprehensive analytical scheme for the analysis of ifosfamide and major metabolites in plasma. STD = standard; IF-Cycl. = cyclic dehydrochlorinated ifosfamide; 4-OHIF(CN)-TMS-Cycl. = cyclic silylated dehydrochlorinated 4-hydroxy-ifosfamide cyanohydrin; N2D-TMS-cycl. = cyclic silylated dehydrochlorinated N2-dechloroethylifosfamide; N3D-TMS-cycl. = cyclic silylated dehydrochlorinated N3-dechloroethylifosfamide; IPM-TMS-Cycl. = cyclic silylated dehydrochlorinated iphosphoramide mustard.

metabolites (including their labeled internal standards) underwent thermal dehydrochlorination. Thus, all of the measured molecular ions represented their respective silylated dehydrochlorinated products and they were selected for monitoring for most of the analytes except two. For the internal standard IF-d_6 and 4-OHIF-d_6, it was found best to use ^{37}Cl isotope ions to avoid any potential overlap with sample ions.

The assay validation and characteristics are summarized in Table 1. As shown, the assay for all of the analytes were linear for at least 40-fold monitored with excellent regression coefficients. The recovery values ranged from 52.5 for 4-OHIF to essentially quantitative for IPM. The within-run CV values ranged from 1.1 to 3.7% at 1 to 5 μg/ml (n = 8) and the between-run CV of 8.1–11.7% (n = 6). The routine sensitivity limits ranged from 0.1 to 0.5 μg/ml, all at 100 μl plasma sample.

3.2. Plasma concentration–time profiles of IF, 4-OHIF, N2D, N3D, and IPM following i.v. administration of IF to the rat

Plasma concentrations of IF, 4-OHIF, N2D, N3D, and IPM following an intravenous bolus administration of IF to the rat at a dose of 40 mg/kg were determined using this assay method as shown in Fig. 5. The true plasma levels of IPM were calculated by the difference between total IPM and 4-OHIF levels, since total IPM levels include the IPM levels generated from 4-OHIF during sample processing. As shown, plasma concentrations of IF declined monoexponentially with an elimination half-life of 43.1 min. The

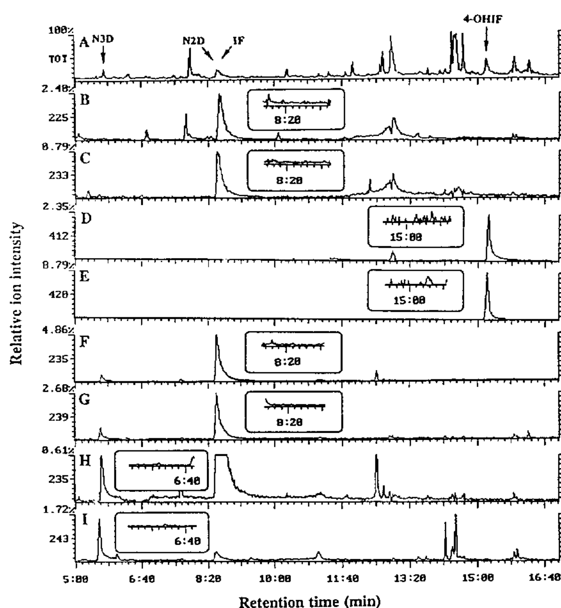

Fig. 3. Representative total and selected-ion GC–MS chromatograms of the derivatives dichloromethane extract residue obtained from a plasma sample from a rat given ifosfamide and blank plasma (insets). (A) Total ion; (B) Selected ion at *m/z* 225 for ifosfamide; (C) Selected ion at *m/z* 233 for ifosfamide-d_6; (D) Selected-ion at *m/z* 412 for 4-hydroxyifosfamide; (E) Selected ion at *m/z* 418 for 4-hydroxyifosfamide-d_6; (F) Selected ion at *m/z* 235 for N2-dechloroethylifosfamide; (G) Selected ion at *m/z* 239 for N2-dechloroethylifosfamide-d_4; (H) Selected ion at *m/z* 235 for N3-dechloroethylifosfamide; (I) Selected ion at *m/z* 243 for N3-dechloroethylifosfamide-d_8.

total clearance and the volume of distribution were 3.1 ml/min and 194.7 ml, respectively. The initial concentration was 61.7 μg/ml and the area under concentration–time (AUC) value was 3833 min · μg/ml. The plasma profiles of all metabolites displayed metabolite formation phase and elimination phase. The levels of 4-OHIF/aldoIF peaked at 17.4 min with a value of 2.38 μg/ml and declined in parallel to those of IF with an apparent half-life of 39.7 min. The AUC value was 241 min · μg/ml. The level of IPM peaked later at 67 min with a maximal concentration of 2.45 μg/ml, and eliminated slower with an apparent half-life of 92 min. The value of AUC was 585 min · μg/ml. The levels of N2D and N3D peaked at 110 min and 118 min, respectively, and both displayed a long elimination phase with apparent half-lives of 136.7 and 144.0 min, respectively. The peak concentrations of N2D and N3D were 2.34 and 1.35 μg/ml, while their AUC values were 829 and 505 min · μg/ml, respectively.

4. Discussion

Although many analytical methods for IF have been published, few included analysis of its metabolites, especially 4-OHIF/aldoIF and IPM probably due to the stability problem. This

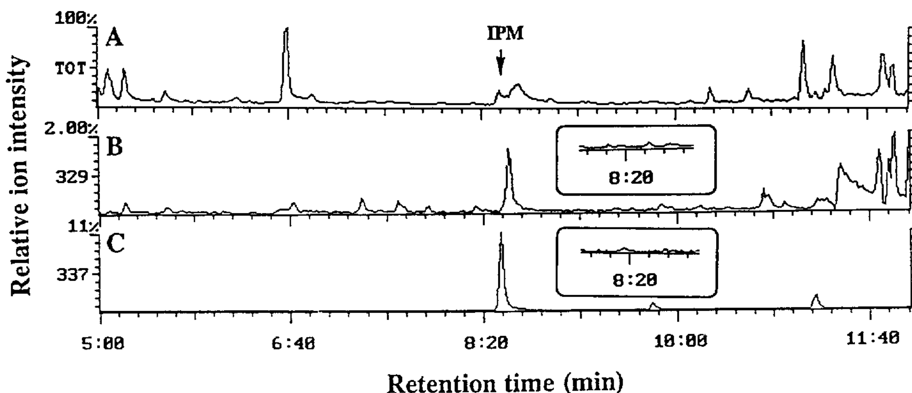

Fig. 4. Representative total and selected-ion GC–MS chromatograms of the derivatized extract residue obtained from solid phase extraction of a plasma sample from a rat given ifosfamide and blank plasma (insets). (A) Total ion; (B) Selected ion at *m/z* 329 for iphosphoramide mustard; (C) Selected ion at *m/z* 337 for iphosphoramide mustard-d_8.

Table 1
Assay validation and characteristics of IF and metabolites

Analyte/I.S.	Ions selected[a] (m/z)	Retention time (min)	Recovery (n = 6)	Linearity range (μg/ml) (r^2)	Within-run (n = 8)		Between-run (n = 6) C.V. (%)	Routine sensitivity limit (μg/ml)
					C.V.(%)	Conc. used		
IF/IF-d_6	225 231, 233[b]	8.6	92.4	0.5–20 (0.999)	3.7	5 μg/ml	10.0	0.5
4-OHIF/4-OHIF-d_6	412 418, 420[b]	15.2	52.5	0.5–20 (0.997)	1.1	5 μg/ml	8.1	0.5
N2D/N2D-d_4	235 239	8.6	60.9	0.1–4 (0.997)	1.7	1 μg/ml	10.3	0.1
N3D/N3D-d_8	235 243	5.7	66.5	0.1–4 (0.997)	4.4	1 μg/ml	11.7	0.1
IPM/IPM-d_8	329 337	8.5	100	0.5–20 (0.997)	1.6	5 μg/ml	8.6	0.5

All analytes underwent thermal dehydrochlorination during derivatization condition.
[a] All ions are MH^+ of the silylated dehydrochlorinated products, except those as indicated.
[b] The ^{37}Cl ion was selected to avoid potential interference from the sample analyte.

problem has now been circumvented by using a stabilization procedure coupled to the use of stable isotopically labeled analogues as the internal standards. These internal standards behave essentially identical to the analytes and compensate procedural losses. Traditionally, for quadruple mass spectrometers a structurally similar compound can be used as the internal standard for a number of analytes, provided that the chromatographic behaviors are sufficiently similar. Thus, CP has been successfully used as the internal standard for the analysis of IF, N2D and N3D [37], and CP-d_8 for both CP and alcophosphamide [46]. However, with the current ion trap mass spectrometer (see Experimental), when structures different from the analytes were used as the internal standards, the results were found less satisfactory. More scattered data and poor linearity were frequently encountered, even though the chemical structures of the internal standards and chromatographic behaviors were similar to the analytes. The reason for this problem is not entirely clear, but may be related to the structure and difference in fragmentation in the ion trap. Subsequently, it was found best to employ deuterium labeled analog as the internal standard for each of the analytes and the results obtained were superior. Although the syntheses of these deuterium analogues required some efforts, the methodologies were readily available as they were related to other projects in our laboratory. Thus, the syntheses of all labeled standards were accomplished exactly as described. The percentage deuterium labels of these compounds ranged from 70% to 98.6%. These % labels were found to be adequate for use as the internal standards since a constant amount for each was used for calibration. In any case, no detectable amount of unlabeled (d_0)

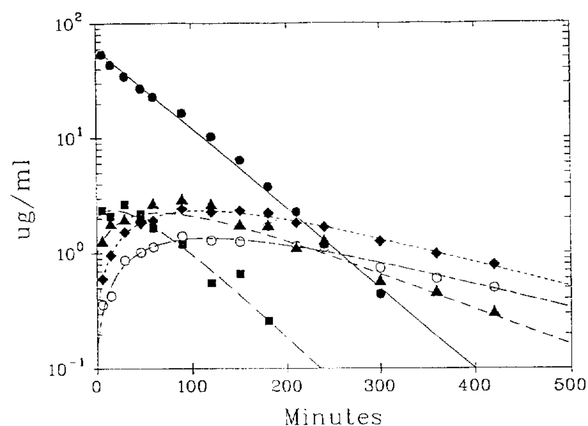

Fig. 5. Plasma concentration–time profiles of ifosfamide and metabolites in a rat following i.v. administration of ifosfamide: ● = IF, ■ = 4-OHIF/aldoIF, ▲ = IPM, ◆ = N2D, ○ = N3D. Lines drawn through these symbols represent best-fitted curves according to appropriate exponential equations.

analyte was found in any of the synthesized labeled internal standards.

In some cases, however, other considerations were needed for the selection of the appropriate internal standard. Initially, N2D-d_6 was synthesized and used as the internal standard for the N2D assay. It was later found a fragment ion of N2D-d_6 at m/z 225 interfered with the analysis of IF for which the ion of the same mass was selected and these two compounds gave the same retention time. Therefore, N2D-d_4 was synthesized and used as the internal standard. No problem was then encountered.

Several reports described the gas chromatographic analysis of IF, N2D and N3D without derivatization [15,17–20,22,23,36,37]. However, under our current GC–MS conditions these species could not be analyzed without derivatization because of their low volability and thermal instability. Silylation was also required for the analysis of 4-OHIF following stabilization to form the cyanohydrin adduct.

In conclusion, a quantitative method for the analysis of IF, 4-OHIF, N2D, N3D and IPM in rat plasma was developed using GC–MS stable isotope dilution techniques. This method has been used to determine simultaneously all three metabolic pathways leading to the antitumor and toxicological effects. This method was found to be sensitive and specific with a lower detection limit of 0.5 μg/ml when 100 μl of plasma was used. This assay method may be used to support routine clinical pharmacokinetic studies of IF.

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