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Determination of 4-hydroxyifosfamide concomitantly with ifosfamide and its dechloroethylated metabolites using gas chromatography and a nitrogen phosphorus-selective detector

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Abstract

A sensitive gas chromatographic (GC)/nitrogen phosphorus detection (NPD) system was developed for the determination of the antitumor drug ifosfamide (Ifos) and its 2-dechloroethylifosfamide (2-Difos), 3-dechloroethylifosfamide (3-Difos) and 4-hydroxyifosfamide (4-OHifos) metabolites in human blood. 4-OHifos was analyzed after coupling with a trapping agent and was used as an indicator of isophosphoramidate mustard (IPM). Ifos and its metabolites 2-Difos, 3-Difos, 4-OHifos and the internal standard (trofosfamide) were extracted into chloroform and then resolved by gas chromatography using a Hewlett Packard HP5 capillary column cross-linked with 5% phenyl methyl silicone (30 m; 530 μm I.D.; 2.65 μm film thickness). Precision and accuracy of the assay were determined over a three-day period and a concentration range of 3.25–50 $\mu\text{g}/\text{ml}$ for Ifos, 0.8–14 $\mu\text{g}/\text{ml}$ for 2D-Ifos, 0.6–10 $\mu\text{g}/\text{ml}$ for 3D-Ifos and 0.08–1.40 $\mu\text{g}/\text{ml}$ for 4-OHifos. The limit of quantitation was set at 3.25, 0.80, 0.62 and 0.08 $\mu\text{g}/\text{ml}$, respectively, for Ifos, 2-Difos, 3-Difos and 4-OHifos. The intra- and inter-day coefficients of variation and accuracies were less than 20%, except for a low concentration 4-OHifos. This assay was then used to provide pharmacokinetic data on antitumor and toxicologic effects following intravenous infusion of Ifos. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ifosfamide; 4-Hydroxyifosfamide

1. Introduction

Ifosfamide (Ifos), like cyclophosphamide, is one of the most important clinical alkylating agent due to its frequent use in cancer chemotherapy either as a single agent or as a component in a combination

regimen. Ifos is currently considered as a therapeutic reference for soft-tissue sarcoma in adults or children [1,2]. It is a chiral prodrug that does not exhibit any cytotoxic activity in vitro, and requires hepatic oxidative metabolism by members of the cytochrome P450 (CYP) superfamily of enzymes in vivo to generate the activated compound 4-hydroxyifosfamide (4-OHifos) [3], which is in equilibrium with its open ring tautomer, aldoifosfamide (Aldoifos). 4-OHifos subsequently yields an alkylating

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isophosphoramidate mustard (IPM), which is regarded as the ultimate intracellular alkylating metabolite [4].

A second Ifo metabolic pathway at one of the chloroethyl groups leads at the formation of 2-dechloroethylifosfamide (2-Difos) and 3-dechloroethylifosfamide (3-Difos), which are the major metabolites of the parent drug. This oxidative pathway,

which is also under cytochrome P-450 dependence [5,6], is responsible for the neurotoxicity of the drug through release of chloroacetaldehyde [7,8]. Consequently, those two pathways (Fig. 1) represent the first step in Ifo metabolism.

Several analytical techniques have been used to quantify Ifo, 2-Difos and 3-Difos, including gas

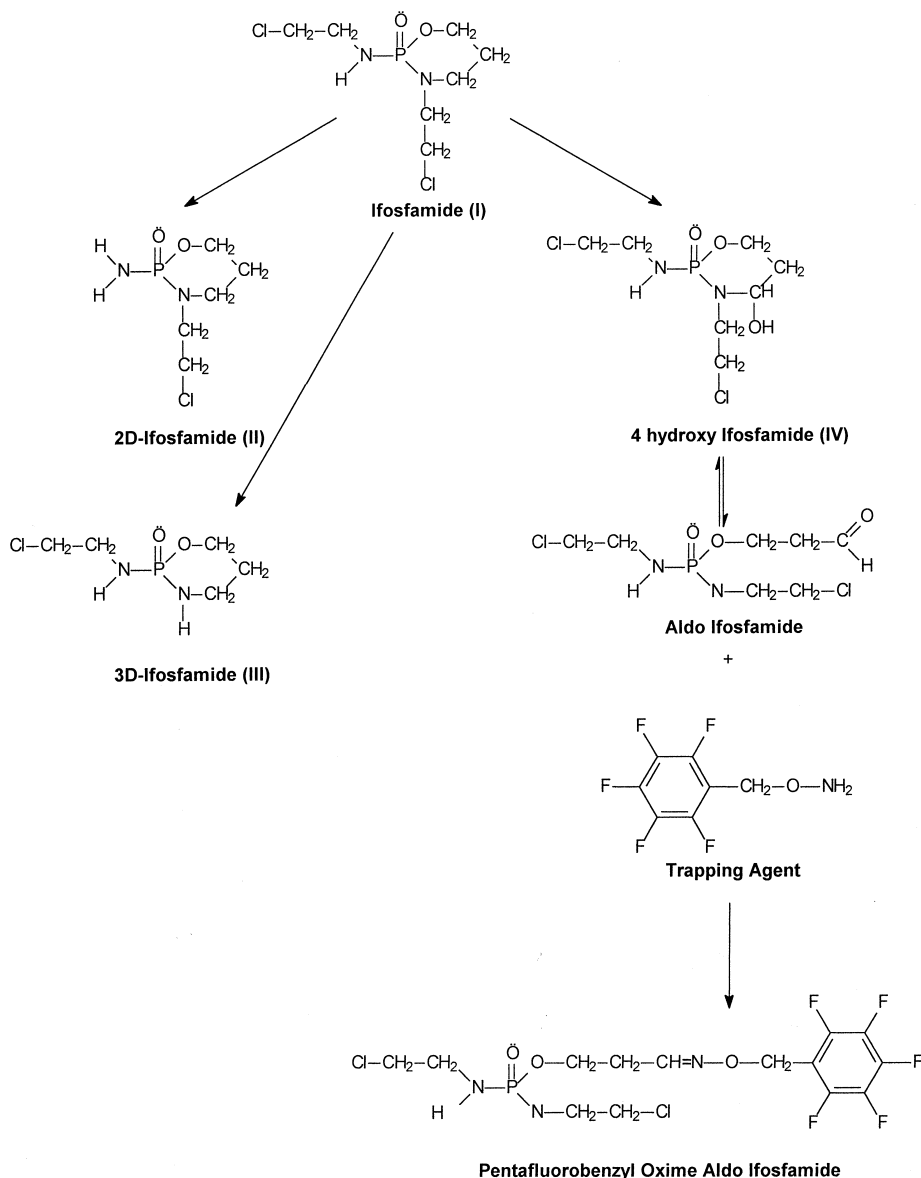


Fig. 1. Chemical structures and schematic metabolic pathway of ifosfamide (I) and its generated metabolites, 2- and 3-dechloroethylated ifosfamide (II–III) and 4-hydroxyifosfamide (IV).

chromatography and mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) or radioactivity methods [9], but few have been applied to 4-OHIfos because of its high instability. In fact, no direct, specific methods are available for this compound in the literature

Thus, we developed a technique of GC–nitrogen phosphorus detection (NPD) to study Ifos and its major metabolites in human blood. 4-OHIfos was analyzed after coupling it with a trapping agent and it was used as an indicator of IPM. Blood levels of Ifos and its metabolites 2-DIfos, 3-DIfos and 4-OHIfos were determined simultaneously with this method, which was then applied successfully in human pharmacokinetic studies following intravenous infusion of Ifos.

2. Experimental

2.1. Reagents

Ifosfamide, the internal standard trofosfamide and the metabolites 2-DIfos, 3-DIfos and 4-hydroperoxy-ifosfamide were generously provided by the Central Analytical Services Department of Asta Group research. The trapping agent *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride and ammonium phosphate were purchased from Sigma (St. Louis, MO, USA). High purity methanol, acetonitrile, chloroform and ethyl acetate were acquired from Merck (Darmstadt, Germany). Sodium chloride, 0.9%, was provided by the Pharmacie Centrale Des Hôpitaux (Nanterre, France).

2.2. Chromatographic system

The determination of Ifos and its metabolites was performed on a Hewlett Packard 5890 gas chromatograph equipped with a nitrogen phosphorus selective detector (NPSD) and a Hewlett Packard GC/SFC 7673 autosampler. The analytical column was a Hewlett Packard HP5 capillary column cross-linked with 5% phenyl methyl silicone (30 m; 530 μ m I.D.; 2.65 μ m film thickness). Helium was used as a carrier gas (flow-rate, 7 ml/min), and hydrogen and air, which were used as detector gases, were at flow

rates of 1.5 and 60 ml/min respectively. The injector temperature was set at 275°C. The oven was programmed to start at 205°C for 9 min and attain its final temperature of 275°C at a rate of 7°C/min. The final temperature was maintained for 5 min. Thus, the total run time was approximately 24 min. NPSD potential was set to obtain a baseline value of 20. Data acquisition was performed using a Hewlett Packard 3396 Series II integrator. The entire system was regulated by a Hewlett Packard 7673 controller.

2.2.1. Gas chromatography–mass spectrometry

GC–MS analyses were performed with a Varian 3400 GC equipped with a Finnigan A 200S GC autosampler operating in the split mode. The mass spectrometer was a Finnigan Mat Model Inco 50 operating in the electron impact and selective-ion monitoring (SIM) mode. Temperatures were set as follows: ion source temperature, 180°C; transfer line temperature, 250°C; electron energy, 70 eV and the emission current, 300 μ A.

The chromatographic separation was performed with a capillary column (Chirasil-Val; 15 m \times 0.30 mm I.D., 0.20 μ m film thickness; Alltech, Heliflex; purchased from Mandel Scientific, Guelph, Canada). GC experimental conditions were as follows: Injection port temperature, 210°C; the GC column temperature was programmed from 170°C for 5 min, increasing by 5.1°C up to 208°C, then was increased to 220°C at a rate 11.0°C and then held for 14 min; helium pressure, 5 p.s.i (33 KPa). The total run time was 28 min.

2.3. Preparation of trapping agent solution

A solution was prepared to trap 4-OHIfos. A 1-g amount of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride was dissolved in 20 ml of methanol to obtain a solution with a final concentration of 50 μ g/ml. Tubes containing 250 μ l of trapping agent solution, together with a solution comprising 1 ml of 2 M phosphate buffer, 3 ml of acetonitrile–methanol (2:1, v/v) and 750 μ l of 0.9% sodium chloride were then prepared. The resulting solution (trapping mix) was gently vortex-mixed. To these tubes were added 1 ml of standard and control solutions as well as blood drawn from patients during clinical assays (between 0.5 and 1.5 ml). For

clinical samples, blood from treated patients was withdrawn directly into a tube containing the trapping mix. This ensured the stability of 4-OHIfos. When blood was drawn directly into the trapping mix, a 24-h contact time at room temperature is needed to ensure a complete trapping action. After this time and following centrifugation, the supernatant was stored until required for further analysis.

2.4. Preparation of diluting solution

Blank blood (1 ml) was drawn in trapping mix tubes to obtain a blank solution for dilutions. The same preparation procedure was used as described above, and the supernatant (trapping blank solution) was stored subsequent use in diluting working solutions and patients' samples.

2.5. Preparation of standard solutions

A stock solution of Ifos was prepared by dissolving an adequate amount of compound in methanol to give a final base concentration of 2 mg/ml. Stock solutions of 2-Difos and 3-Difos were prepared to give a final concentration of 2 mg/ml for each product. These solutions remained stable for two months at -20°C .

An internal standard stock solution was obtained by dissolving an adequate amount of trofosfamide in methanol to give a final base concentration of 2 mg/ml. The solution then remained stable for at least two months at -20°C .

Because of the high instability of 4-OHIfos, the stock solution of this compound was obtained prior to preparation of standard samples. An adequate amount of 4-hydroperoxyifosfamide was dissolved in a solution containing 0.9% sodium chloride, and 4-OHIfos was then generated spontaneously to give a final concentration of 1 mg/ml.

2-Difos and 3-Difos stock solutions (100 μl each) were added to 600 μl of Ifos stock solution. After vortex-mixing, the solution was dried down under nitrogen before the addition of 19.5 ml of blank human blood. The mix was then vortex-mixed for 5 min. A 500- μl volume of a 4-OHIfos solution was then added to the previous solution. Final Ifos, 2-Difos, 3-Difos and 4-OHIfos concentrations were 520, 140, 100 and 14 $\mu\text{g}/\text{ml}$, respectively. Aliquots

(1 ml) of this solution were added to the trapping mix and were extensively vortex-mixed. After 24-h contact, the tubes were centrifuged and the supernatant was stored at -20°C . Under these conditions, supernatant is stable for at least six months.

Thus, six different levels of standard (G6 to G1) were prepared for each compound investigated by diluting the working solution with blank trapping solution. The higher level (G6) was prepared by diluting a tenth the working solution. Standards G4 to G1 were then prepared from G6 by twofold serial dilution using the blank trapping solution, and G5 was prepared by diluting G6 3:4 (v/v). The final concentrations of the analytes for each point in the standard curve are indicated in Table 1.

2.6. Preparation of quality control solutions

Working quality control solutions were obtained by using the same preparation procedure as described for standards. Three levels were then prepared by diluting the working solutions with adequate amounts of blank trapping solution and these were included in validation and sample batch analysis. Controls were prepared in order to obtain similar concentrations to those used for standard solutions G1, G3 and G6. The concentrations of these control solutions are given in Table 2.

2.7. Sample preparation

For samples from patients, tubes containing the trapping mix were weighed before and after blood was withdrawn. Calculus was processed in order to quantify amount of blood which was drawn on the basis that 1 ml of whole blood weighs 1 g. This allowed us to provide results per ml with respect to the total blood drawn, by multiplying the different concentrations calculated by this difference of weight.

Patients' samples, and standard and control samples were processed in the same way. The trofosfamide (I.S.) working solution (100 μl) was added to 500 μl of an unknown sample. Chloroform (2 ml) was then added as a liquid extraction step. Samples were mixed for 2 min using a vortex-mixer. After ultracentrifugation (speed, time), the organic phase was kept and placed in an evaporator (50°C) where it

Table 1

Back-calculated and precision data for ifosfamide and metabolites obtained from standard curve concentrations over a four-day period (concentrations in $\mu\text{g/ml}$)

Nominal	Mean	S.D.	RSD (%)	M.R.E. (%)	Nominal	Mean	S.D.	RSD (%)	M.R.E. (%)
Ifosfamide					2-Dechloroethylated ifosfamide				
3.25	2.92	0.171	5.70	-10.1	0.80	0.76	0.061	8.25	-4.69
6.50	6.53	0.310	4.81	0.38	1.75	1.67	0.120	7.05	-4.71
13.0	13.1	0.232	1.72	1.06	3.50	3.52	0.053	1.50	0.43
26.0	26.2	0.993	3.77	0.90	7.00	7.10	0.302	4.18	1.43
39.0	39.2	0.360	0.92	0.62	10.5	10.7	0.180	1.67	1.93
52.0	51.7	0.302	0.59	-0.61	14.0	13.8	0.214	1.50	-1.34
3-Dechloroethylated ifosfamide					4-Hydroxy ifosfamide				
0.62	0.55	0.142	25.2	-10.9	0.08	0.07	0.013	19.9	-18.7
1.25	1.34	0.200	15.2	7.20	0.17	0.16	0.024	14.4	-2.94
2.50	2.46	0.073	2.69	-1.70	0.35	0.38	0.029	7.75	8.57
5.00	5.00	0.224	4.36	0.05	0.70	0.69	0.077	11.1	-0.36
7.50	7.71	0.321	4.17	2.77	1.05	1.04	0.007	0.68	-0.48
10.0	9.80	0.202	2.05	-2.00	1.40	1.39	0.037	2.65	-0.36

was dried under a flow of nitrogen. Ethyl acetate (150 μl) was then added and the samples were vortex-mixed. The injected volume ranged from 0.5 to 1.5 μl .

2.8. Validation

Calibration curves were obtained prior to each batch analysis. Quality controls were included to validate every calibration curve used and to ensure sample stability during analysis. Calibration curves were obtained by non-weighted least-squares linear regression analysis of the peak-area ratios of each investigated compound/trophosphamide (I.S.) versus the concentration of the investigated drug. Calibra-

tion curve equations were used to calculate the concentrations of Ifos, 2-Difos, 3-Difos and 4-OHifos in samples and quality controls from their peak-area ratios.

3. Results and discussion

3.1. Trapping agent-4-OHifos coupling reaction specificity

4-OHifos is generally considered to be in equilibrium with its open ring tautomer, aldoifos, as described in Ifos metabolic pathways. The purpose of the trapping agent is to generate a pentafluorobenzyl

Table 2

Inter-day accuracy and precision data for ifosfamide and its metabolites ($n=10$)^a

Nominal	Mean	S.D.	RSD (%)	M.R.E. (%)	Nominal	Mean	S.D.	RSD (%)	M.R.E. (%)
Ifosfamide					2-Dechloroethylated ifosfamide				
3.25	2.79	0.171	6.09	14.1	0.80	0.64	0.070	0.13	20.0
13.0	13.1	0.293	2.22	0.60	3.50	3.47	0.081	2.30	8.57
52.0	51.8	1.021	1.97	-0.46	14.0	13.6	0.412	3.00	-3.14
3-Dechloroethylated ifosfamide					4-Hydroxyifosfamide				
0.62	0.70	0.071	10.0	-11.4	0.08	0.07	0.021	28.6	-12.5
2.50	2.48	0.131	12.1	-8.00	0.35	0.36	0.052	13.9	2.85
10.0	9.75	0.370	3.80	-2.50	1.40	1.36	0.070	5.11	-2.85

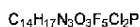
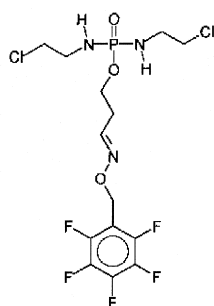
^a Concentrations are given in $\mu\text{g/ml}$.

oxime aldoifosfamide, which has already been studied for cyclophosphamide [10]. This chemical reaction allows the trapping of the aldoifos form present in the blood as well as of aldoifos as soon as it is generated from unstable 4-OHifos. This chemical process is performed at ambient temperature over a 24-h period. The resulting oxime is shown in Fig. 2.

It would appear that 2 M ammonium phosphate buffer generates the optimum pH for stabilizing oxime as it is formed. GC–MS was used to determine the type of compound generated. Confirmation of the chemical identities of the oxime derivative of aldoifosfamide and any reaction between the trapping agent and other analytes, Ifos, 2D-Ifos and 3D-Ifos, was performed in collaboration with the Wainer group.

3.1.1. Mass spectrometry

Typical GC–MS chromatograms relevant to the chemical reaction of pentafluorobenzyl oxime aldoifosfamide and the enantiomers of ifosfamide, the dechloroethylated metabolites and the internal standard are shown in Figs. 3 and 4, respectively. The corresponding mass spectra of these compounds are shown in Figs. 3B and 5, respectively. The simultaneous presence of fragment ions at m/z 393/395 (loss of a $\text{NHCH}_2\text{CH}_2\text{Cl}$), m/z 274 (loss of a $\text{C}_7\text{H}_2\text{F}_5\text{O}$), m/z 252 ($\text{C}_4\text{H}_{10}\text{Cl}_2\text{N}_2\text{PO}_2$), m/z 221/222 (loss of a $\text{C}_{10}\text{H}_7\text{F}_5\text{NO}$) and the molecular peak



Relative Molar Mass : 472,17
Molecular Weight : 471,030476
Percentage Composition : C35,6% H3,6% N8,9% O10,2% F20,1% Cl 15,0% P6,6%

Pentafluorobenzyl Oxime Aldo Ifosfamide

Fig. 2. Oxime formed after coupling of trapping agent and 4-hydroxyifosfamide.

m/z 181 (loss of a $\text{C}_7\text{H}_{15}\text{Cl}_2\text{N}_3\text{PO}_2$) permits the confirmation of pentafluorobenzyl oxime aldo ifosfamide. (*R,S*)-Ifos, (*R,S*)-2- and 3-Difos and Trofo (I.S.) produced a molecular base peak at m/z (211), 149 and 273 (loss of a CH_2Cl), respectively. The molecular base peaks for (*R,S*)-Ifos, (*R,S*)-2- and 3-Difos and Trofo are similar to those from previously reported studies [11].

3.2. Specificity of chromatographic analysis

Fig. 6D displays a typical chromatogram obtained from a single injection of Ifos, 2-Difos, 3-Difos, 4-OHifos and the internal standard trophosphamide. The 2D metabolite was eluted first and then 3Difos and Ifos, the internal standard and finally 4-OHifos. Because of the low levels of 4-OHifos present in samples, it was necessary to increase NPD sensitivity immediately after trophosphamide (I.S.) elution. Retention times for Ifos, 2-Difos, 3-Difos, 4-OHifos and trophosphamide were 11.6, 5.3, 6.3, 21.9 and 16.0 min, respectively.

Four different experiments were performed to assess the selectivity of the chromatographic method displayed here:

- When the injection of 4-OHifos working solution was processed, no peak was present on the chromatogram because of the high instability of the compound (Fig. 6A).
- Fresh 4-OHifos working solution was prepared following the addition of the trapping agent. The chromatogram showed one peak with a retention time of 21.9 min (Fig. 6B).
- To demonstrate that there were no interferences between the trapping agent and other analytes, working solutions of Ifos and both dechloroethyl metabolites were prepared and the trapping agent was added. After injection, the chromatogram showed no evidence of co-elution of the trapping agent with any other analytes and vice-versa (Fig. 6C).
- When a regular solution of all analytes and the trapping agent was prepared and injected, each compound was eluted at the expected retention time. No interference was found between chromatogram peaks (Fig. 6D).

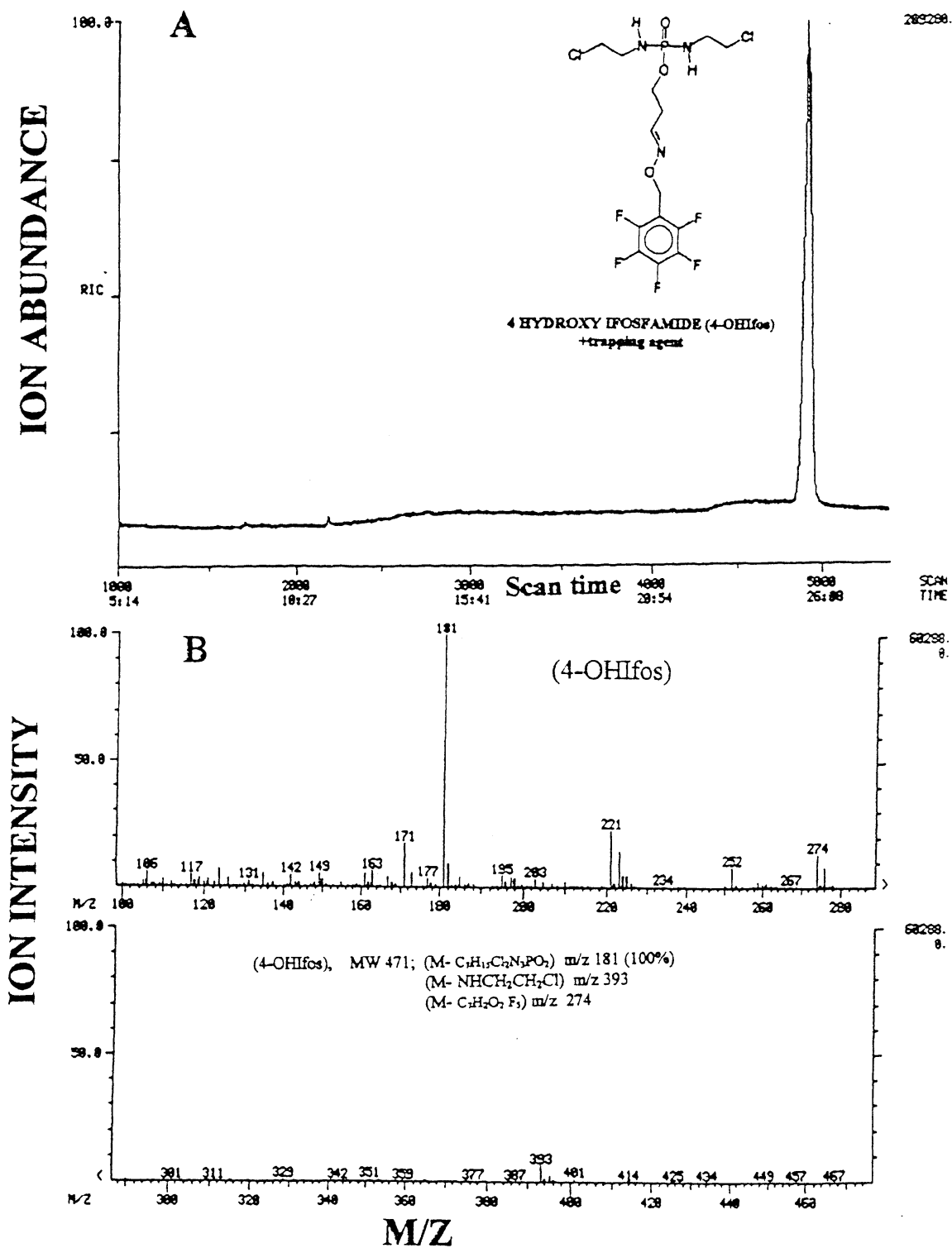


Fig. 3. Reconstituted total ion chromatogram (RIC) (A) and EI mass spectrum (B) of 4-OHifos solution with trapping agent.

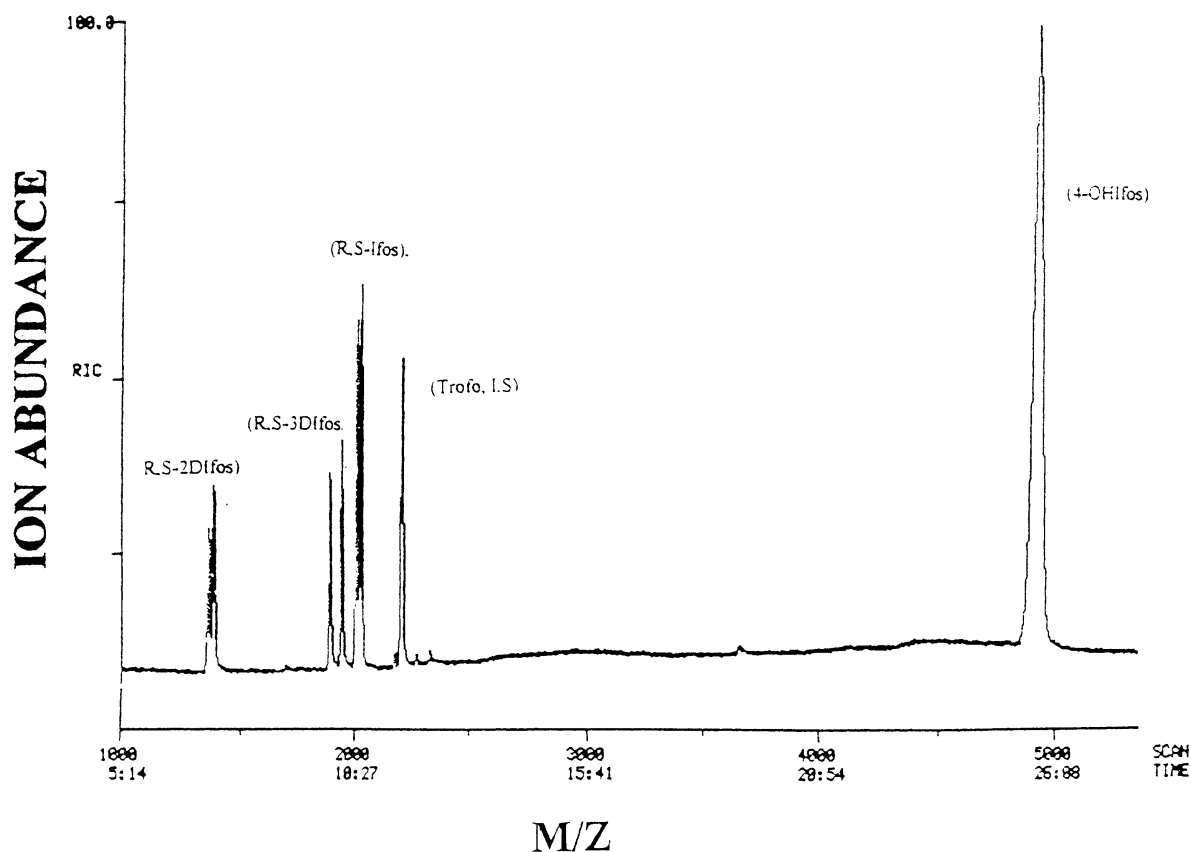


Fig. 4. Reconstituted total ion chromatogram of the enantiomers of Ifos, 2DIfos, 3DIfos and Trofo (I.S.) solution at 10 $\mu\text{g/ml}$ with trapping 1=(*S*)-2DIfos, 2=(*R*)-2DIfos, 3=(*S*)-3DIfos, 4=(*R*)-3DIfos, 5=*S*-Ifos, 6=*R*-Ifos, 7=Trofo (I.S.) and 8=4-OHIfos.

Analysis of drugs from the blank trapping solution indicated that injection of diluting solution caused no interference with analytes and internal standard over the concentration range described here.

Thus, the trapping agent was essential for 4-OHIfos quantification. No interferences between the trapping agent and other analytes were noted. No interferences have been observed with co-medication administered in this type of patient.

3.3. Linearity and sensitivity

Correlation coefficients for each analyte's calibration curve were within the range of 0.999 to 1 for Ifos, 0.998 to 1 for 2-DIfos, 0.996 to 0.999 for 3-DIfos and 0.993 to 0.998 for 4-OHIfos. Linear responses were obtained in the 3.25–52.0 $\mu\text{g/ml}$

range for Ifos, 0.80–14.0 $\mu\text{g/ml}$ for 2-DIfos, 0.62–10.0 $\mu\text{g/ml}$ for 3-Difos, and 0.08–1.40 $\mu\text{g/ml}$ for 4-OHIfos. These results indicated that the linear response was adequate for the method and the analytical process.

Precision, as measured by the relative standard deviations (RSD) at each of the spiked analyte concentrations and evaluated by the mean of concentrations back-calculated from the respective calibration curves, are shown in Table 1 for different analytes over a four-day period.

3.4. Accuracy and precision

The accuracy of the method was established by a three-day validation process. For each analyte, accuracy was then determined by comparing the means

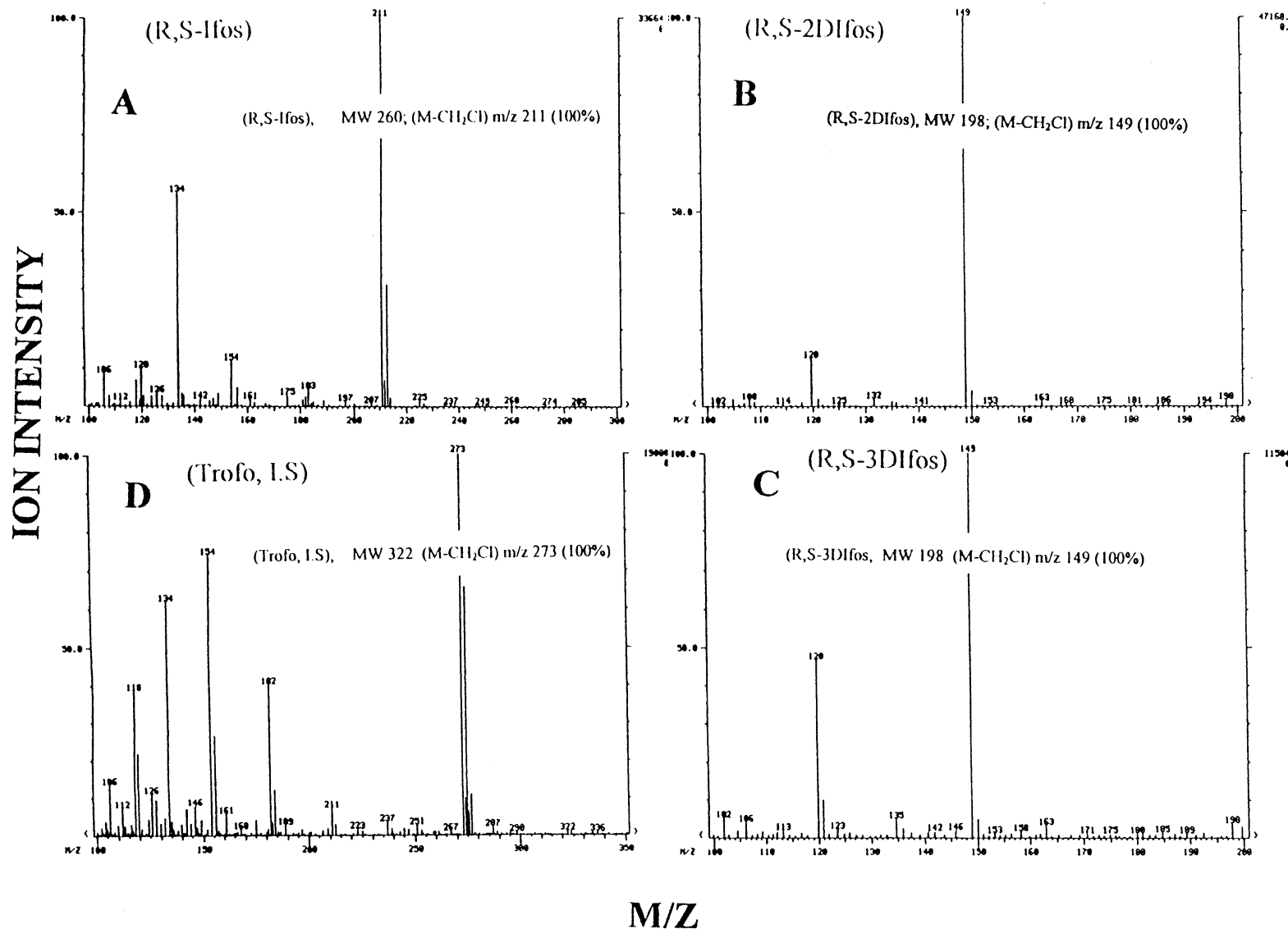


Fig. 5. EI mass spectrum of the enantiomers of Ifos, 2difos, 3difos and Trofo. A=(R,S)-2DIfos, B=(R,S)-3DIfos, C=(R,S)-Ifos and D=Trofo (I.S.).

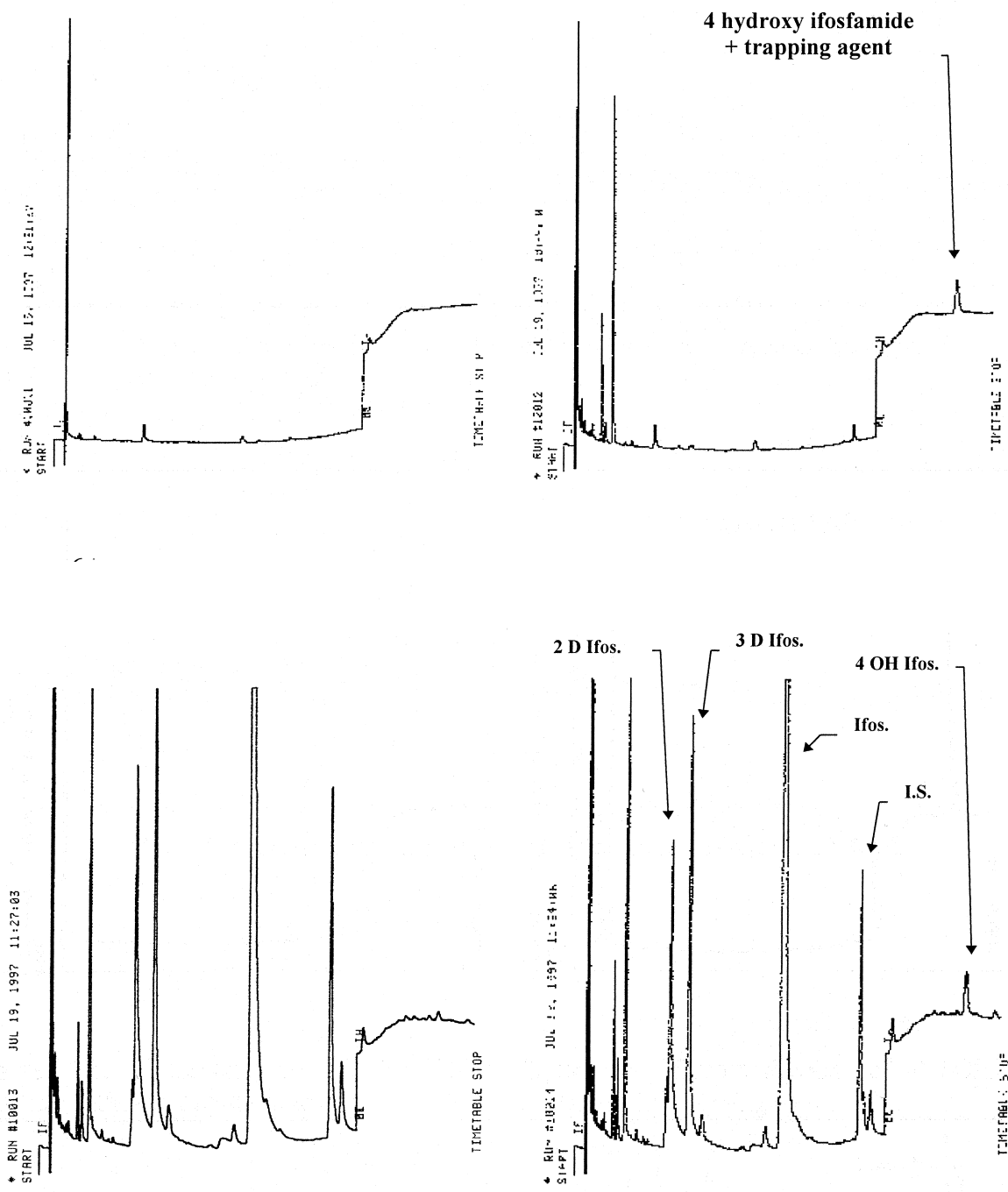


Fig. 6. Chromatograms obtained after experiments A, B, C and D.

of measured concentrations with the nominal concentration for three levels of quality control solutions. All overall and daily C.V.s and the mean

residual error (M.R.E.) were below fixed limits of 25%. Detailed results for each analyte are reported in Table 2.

3.5. Quantification limit

The quantification limit (QL) was set at 3.25, 0.80, 0.62 and 0.08 $\mu\text{g/ml}$ for Ifos, 2-DIfos, 3-DIfos and 4-OHIfos, respectively. Ten replicates at the lowest standard level were analyzed to evaluate the QL for each analyte. The QL was fixed at the lowest concentration of QC where the C.V. and the M.R.E. were demonstrated to be below 30 and 25%, respectively. The results are summarized in Table 3.

The QL of the Ifos was determined under these conditions with respect to quantification of 4-OHIfos. Under other conditions (no quantification of metabolites), this value is lowest, being in the same range as 2-DIfos.

The QL of the presented method for Ifos and its dechloroethylated metabolites is in the same range as that found using other GC methods, as described in the literature, such as GC–MS detection [12] or lowest than others using a flame ionization detection method [13].

3.6. Applications

The present method was applied to several pharmacokinetic studies in humans. The concentration range of analytes allowed good exploration of the pharmacokinetic processes after Ifos administration in protocols for both adults or children. The QL enabled us to provide a good estimate of the distribution and elimination phases of Ifos and its metabolites. These studies were performed to determine the best means of administering the drug

through comparison of toxic and antitumor pathways [14]. The typical kinetic profiles obtained are shown in Fig. 7. These data were obtained after a continuous infusion of the parent compound over seven days at a dose of 3 $\text{g/m}^2/\text{day}$. Levels of Ifos and its metabolites under these conditions of administration were in the range of the method and above the QL.

4. Conclusion

Although various analytical methods allow simultaneous quantification of Ifos and its two dechloroethylated metabolites, few permit easy and rapid determination of 4-OHIfos in blood, due to the high instability of this compound. This problem was overcome here by using the trapping agent *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride as a stabilizer.

The GC–NPD method presented here allowed the direct analysis of Ifos and its metabolites without any need for derivatization. The addition of the trapping agent enabled us to quantify 4-OHIfos levels in blood. A small amount of blood can be used since the NPD is highly sensitive, thereby facilitating evaluation of the pharmacokinetics of Ifos and its metabolites during long-term protocols. Cross-validation between the GC–NPD and GC–MS methods ensured the accuracy and precision of the technique and also enabled us to identify the quantified compounds.

In summary, a quantitative method for the analysis of Ifos, 4-OHIfos, 2-DIfos and 3-DIfos in human

Table 3
Intra-day accuracy and precision data for ifosfamide and its metabolites ($n=6$)^a

Nominal	mean	S.D.	RSD (%)	M.R.E. (%)	Nominal	mean	S.D.	RSD (%)	M.R.E. (%)
Ifosfamide					2-Dechloroethylated ifosfamide				
3.25	3.38	0.141	4.00	0.04	0.80	0.81	0.062	7.41	1.25
13.0	13.8	0.062	0.43	6.51	3.50	3.64	0.053	1.37	4.00
52.0	53.2	0.671	1.26	2.31	14.0	14.3	0.173	1.21	2.14
3-Dechloroethylated ifosfamide					4-Hydroxy ifosfamide				
0.62	0.59	0.043	6.10	−4.84	0.08	0.08	0.010	16.2	5.00
2.50	2.64	0.032	1.17	5.60	0.35	0.36	0.021	5.55	2.85
10.0	10.4	0.412	3.92	4.00	1.40	1.36	0.071	5.00	−2.86

^a Concentrations in $\mu\text{g/ml}$.

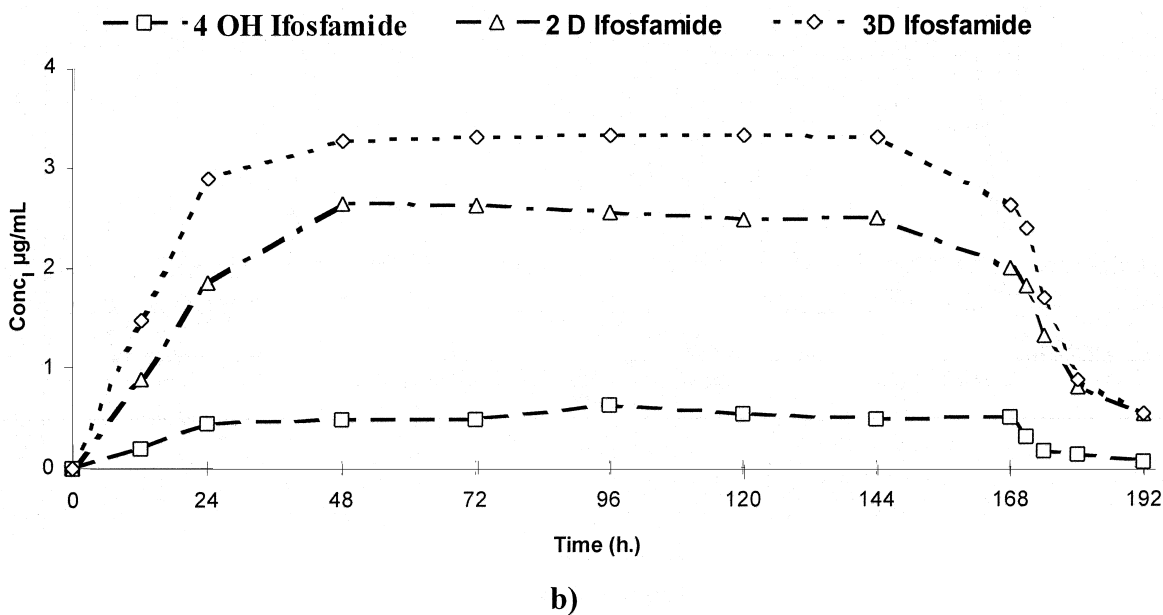
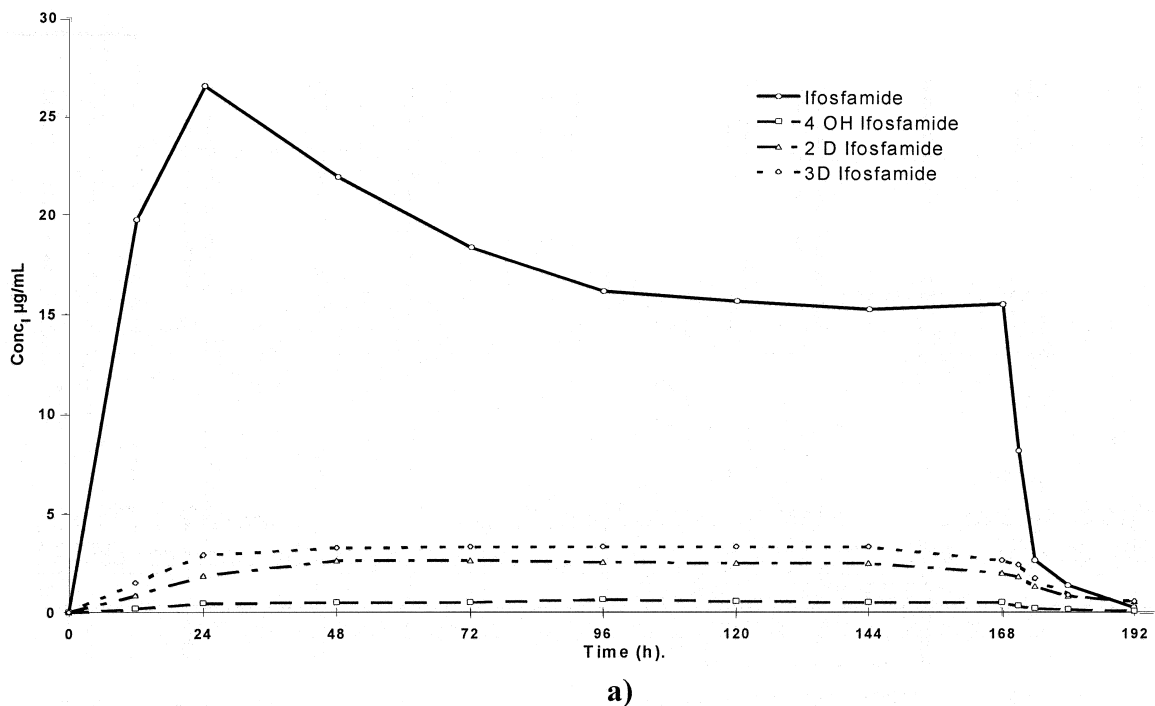


Fig. 7. Kinetic profiles of ifosfamide and its metabolites after a continuous infusion in children.

blood was developed using GC–NPD. This method was used for the simultaneous determination of all three metabolic pathways leading to antitumor and toxicologic effects. It was found to be sensitive and specific, showing a lower sensitivity level than described previously. This assay method may be used in conjunction with routine clinical pharmacokinetic studies of Ifos. The method is suitable for the determination of Ifos and metabolites in children because only very little blood is needed.

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