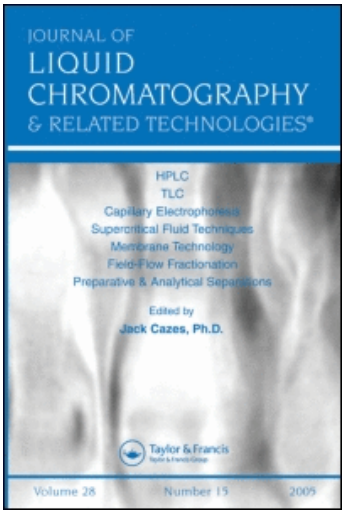

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SIMULTANEOUS DETERMINATION OF IFOSFAMIDE AND ITS METABOLITE IFOSFORAMIDE MUSTARD IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Online publication date: 15 November 2000

To cite this Article Kerbusch, T. , Huitema, A. D. R. , Jeuken, M-J. J. , Derraz, J. , Tibben, M. M. and Beijnen, J. H.(2000) 'SIMULTANEOUS DETERMINATION OF IFOSFAMIDE AND ITS METABOLITE IFOSFORAMIDE MUSTARD IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 23: 19, 2991 — 3010

To link to this Article: DOI: 10.1081/JLC-100101838

URL: http://dx.doi.org/10.1081/JLC-100101838

J. LIQ. CHROM. & REL. TECHNOL., 23(19), 2991–3010 (2000)

SIMULTANEOUS DETERMINATION OF IFOSFAMIDE AND ITS METABOLITE IFOSFORAMIDE MUSTARD IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Because ifosforamide mustard (IFM) is the active alkylating metabolite of ifosfamide (IFO) it is of particular interest in the pharmacokinetic analysis of patients undergoing IFO treatment. This paper presents an assay for the simultaneous determination of IFM and IFO after derivatization with diethyldithiocarbamate (DDTC), subsequent liquid-liquid extraction of the plasma with acetonitrile (AcN) and using reversed phase high performance liquid chromatography (RP-HPLC) with ultra-violet (UV) detection at 276 nm. Structural confirmation of the analytes was accomplished using mass spectrometry (MS).

Reaction conditions such as incubation duration, temperature, and concentration of derivatization agent were investigated; 30 min at 70°C with 100 mg/mL DDTC was optimal. The presented analytical method proved to be accurate, precise, and linear for IFM and IFO concentrations, ranging from 0.100-50.0 and 0.100-100 μg/mL, respectively, and with lower limits of quantitation of 0.100 μg/mL for both analytes.

A typical patient pharmacokinetic profile is presented to demonstrate the applicability of the assay in clinical samples. The analytical method could be employed in high-throughput clinical analysis of IFM and IFO patient samples.

INTRODUCTION

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide (IFO, Holoxan, Ifex), is commonly used for the treatment of various solid tumors, soft tissue sarcomas, and hematological malignancies in adults and children. IFO is a prodrug which needs activation via the cytochrome P450 enzymatic system in the liver to exert its alkylating activity.[1]

First IFO is hydroxylated to 4-hydroxyifosfamide (4-OHIF), as represented in Figure 1. 4-OHIF exists in equilibrium with its tautomeric form, aldoifosfamide (AldoIF). AldoIF can be converted into the final alkylating agent ifosforamide mustard (IFM) with concurrent acrolein formation.

Besides activation, de-activation occurs, yielding 2-dechloroethyl-ifosfamide (2-DCEIF) and 3-dechloro-ethylifosfamide (3-DCEIF) with equimolar release of neurotoxic chloroacetaldehyde. In the (tumor) cell IFM reacts with DNA causing interstrand cross-links,[2] leading to cell death. IFO is known to increase its own metabolism (autoinduction).[3]

Recently, an assay was reported for IFM.[4] This method consisted of direct derivatization of IFM in plasma with diethyldithiocarbamate (DDTC) and subsequent solid phase extraction (SPE) of the resulting derivative. The samples were stabilized by adding semicarbazide (SCZ) and sodium chloride (NaCl), which prevent auto-catalytic breakdown of 4-OHIF to IFM and further IFM breakdown, respectively.

During large scale implementation of this method various problems were encountered. These were mainly, clogging of the SPE columns, low analytical column half-life, and late co-eluting peaks, preventing rapid use in clinical investigations with high sample throughput. A faster, more reproducible, method has now been developed for IFM, which also permitted simultaneous determination of IFO.

No easy to use assay for both IFM and IFO by HPLC has been described thus far. Derivatization of IFM and IFO with DDTC leads to IFM(DDTC)$_2$ and IFO(DDTC), as depicted in Figure 2. Structural confirmation of the analytes and assay validation were performed for both IFM and IFO.

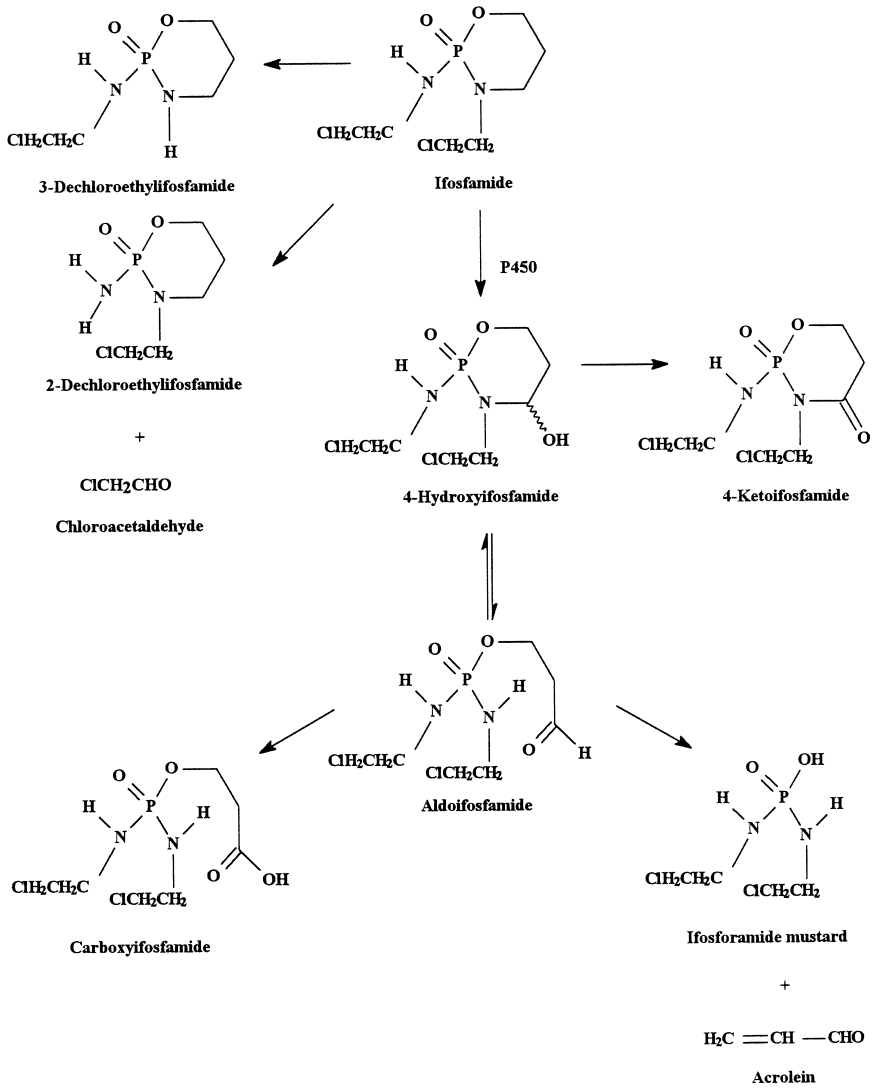

Figure 1. Metabolism of ifosfamide.

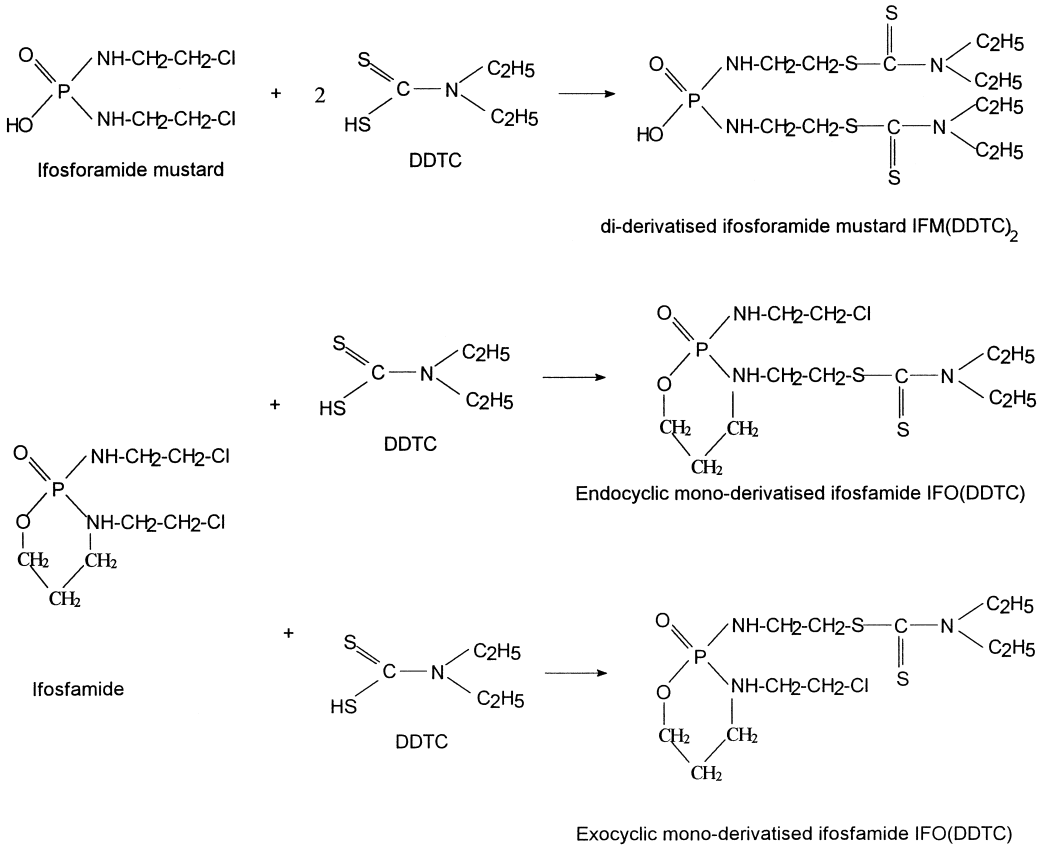

Figure 2. Derivatization of ifosforamide mustard and ifosfamide with diethyldithiocarbamate (DDTC).

EXPERIMENTAL

Equipment

The HPLC equipment consisted of an integrated system with a model SCM1000 in-line degassing unit, a model P1000 pump, a model AS3000 automated injector with a 100-µL loop, a model UV150 ultra-violet (UV) detector operated at 276 nm, and a DataJet integrator (Thermo Separation Products, Breda, The Netherlands).

Data was analyzed using PC1000 software (Thermo Separation Products, Breda, The Netherlands) using a Dell optiplex Gs Pentium personal computer. A 250 x 40 mm I.D. RP8 5 µm particle size Prodigy 5 C8 column with a Security Guard C8 pre-column (Phenomenex, Torrance, CA, USA) was operated at ambient temperature. The analytical column was washed monthly with 1-propanol by which a high separation efficiency was maintained.

The mobile phase consisted of acetonitrile (AcN):water (32:68 v/v) containing 25 mM K_2HPO_4 buffer pH 8.0. For identification of the derivatives, a mass spectrometer (MS) was used operating with direct continuous split-injection (Sciex, Thornhill, ON, Canada). Electrospray ionization (1500 V) was achieved using a TurboIon™ sample inlet. Ions were created at atmospheric pressure and were transferred to a model API 365 triple quadrupole mass spectrometer (LC/ESI-MS/MS) with a dwell-time of 0.1-1 msec. The channel electron multiplier was set at 1900 V.

For optimizing the wavelength of detection, the UV-absorption spectrum was recorded on-line using a Waters model 996 Photodiode-Array (PDA) detector (Waters Chromatography, Milford, MA, USA).

Chemicals

IFM (D-18847, Lot 034.5333) and all other IFO and cyclophosphamide metabolites were the kind gifts of Dr. J. Pohl, ASTA Medica AG (Frankfurt, Germany). Sodium diethyldithiocarbamate was obtained from Sigma (St. Louis, MO, USA). AcN and methanol (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands).

K_2HPO_4, sodium chloride, hydrochloric acid and sodium hydroxide (suprapure grade) were obtained from Merck (Darmstadt, Germany). Semicarbazide hydrochloride (p.a. grade) was purchased from Acros (Geel, Belgium). Distilled water was used throughout.

Preparation of Standards

Blank, drug-free plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Plasma was centrifuged at 1,000 g for 5 min and the pellet was discarded. To the supernatant, a solution containing 2 M SCZ, 1 M NaCl pH 7.40 (SCZ*) was added, yielding a mixture of plasma:SCZ* 10:1 v/v (plasma*). After storage of at least 1 hour at -70°C the plasma* was thawed and centrifuged again at 1,000 g for 5 min. The supernatant was used as blank plasma in the assay. IFM was dissolved as a 1 mg/mL solution in a phosphate buffer (1 M NaCl, 0.1 M K_2HPO_4, pH=8.00)(PPB), immediately prior to preparation of the standards.

IFO was also dissolved as a 1 mg/mL solution in PPB. To 500 µL plasma*, 350 µL IFM and IFO solutions in PPB were added in a 2.0 mL Eppendorf cup, resulting in combined calibration curves of 0, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, and 50.00 µg/mL IFM and 0, 0.100, 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, and 100 µg/mL IFO.

Quality control samples were prepared similarly but from different stock solutions and separate weighting of the standards at 0.100, 0.250, 5.00, and 50.0 µg/mL IFM and 0.100, 0.500, 50.0, and 100 µg/mL IFO.

Sample Pretreatment

The derivatizing agent was added as a 50 µL volume of 100 mg/mL DDTC in PPB to 500 µL plasma* and 350 µL PPB. After whirl-mixing for 15 sec, the samples were placed for 30 min at 70°C in a thermostatically controlled water-bath. After incubation, the samples were placed on ice-water in order to stop the derivatization reaction.

Liquid-liquid extraction was performed by adding 1,000 µL AcN. After whirl-mixing thoroughly three times for 1 min and subsequent centrifugation at 3,000 g for 10 min at 4°C, a 700 µL volume of the AcN fraction was transferred to a clean 1.5 mL Eppendorf cup and evaporated to dryness under a gentle stream of nitrogen at 40°C.

The samples were reconstituted by adding 150 µL mobile phase. After whirl-mixing for 1 min and subsequent centrifugation at 3,000 g for 5 min at ambient temperature, the supernatant of the samples were transferred to 200 µL inserts in HPLC vials. The injected volume was 50 µL.

Patient Sample Collection and Pretreatment

Whole blood samples were collected and immediately placed on ice-water, centrifuged at 1,000 g for 5 min at 4°C, and 1 mL plasma was transferred to a

2-mL Eppendorf cup containing 100 µL SCZ*, yielding plasma*. After thorough vortex mixing, the samples were stored at -70°C. The entire sample handling was always performed within 10 min. After thawing, a 500 µL volume of patient plasma* was prepared in duplicate, identical to standard sample pretreatment.

Identification

Identification of derivatized IFM was achieved by derivatizing an 850 µL volume of 5 mg/mL IFM in PPB with 50 µL 500 mg/mL DDTC in water and incubation, and extraction with AcN as described for the plasma samples. After evaporation of the organic extract, the sample was reconstituted in 100 µL mobile phase and injected in the HPLC system.

The analyte was isolated by collecting the fraction containing the IFM-derivative. The eluate was again extracted with 2:1 (v/v) AcN after addition of 1 g NaCl. After whirl-mixing and centrifugation, the AcN-layer was transferred and evaporated, as described above. Since the residue could be contaminated by NaCl dissolved in co-extracted water in the AcN-layer, the dry residue was again extracted with 1 mL AcN. After transfer of the AcN-layer and subsequent evaporation, the clean residue was stored at -70°C pending identification by MS.

Derivatization of IFO yields two products, as represented in Figure 2, with two corresponding peaks in the HPLC chromatogram. The IFO-derivatives were isolated similarly, by collecting both peaks separately. Partial re-injection of the final AcN-layer on the HPLC system confirmed the purity of the isolated IFM and IFO-derivatives. The isolated derivatives were reconstituted in 80% methanol solution and analyzed by MS for identification.

Optimization of Derivatization

Derivatization conditions were changed to find the optimal incubation temperature, duration, and DDTC concentration. Variations in conditions were tested with 0.500 and 5.00 µg/mL IFM spiked plasma in duplicate. Derivatization temperature was investigated at ambient temperature (20°C), 40°C, and 70°C for 5, 10, 30, and 60 min, and the reaction duration at 70°C for 0, 5, 10, 20, 30, 60, and 120 min.

To find the optimal derivatization reagent, concentration tests were performed at 70°C for 30 min with 0, 0.100, 0.500, 1.00, 5.00, 10.0, 50.0, 100, and 500 mg/mL DDTC.

Specificity and Selectivity

Potential interference from endogenous compounds was investigated by the analysis of six different blank plasma samples. The following compounds were investigated for interference with the analytical method: 2-DCEIF, 3-DCEIF, didechloroethylifosfamide, 4-ketoifosfamide, carboxyifosfamide, 4-OHIF with and 4-OHIF without, semicarbazide stabilization. Possible co-medication was also tested for interference. Tested substances were topotecan, paclitaxel, ketoconazole, granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, caffeine, furosemide, and sodium 2-mercaptoethane sulphonate (MESNA). All compounds were tested at a concentration of 20 μg/mL.

Limit of Quantitation

The lower limits of quantitation (LLQ) of IFM and IFO were investigated in plasma samples from three different donors, by a five-fold determination in three analytical runs. For the concentration to be accepted as the LLQ, the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) were to be less than 20%. The upper limits of quantitation (ULQ) of IFM and IFO were defined as 50.0 and 100 μg/mL, respectively, based upon the expected clinical concentration range.

Validation: Accuracy, Precision, and Linearity

Accuracy, as well as between-day and within-day precision of the method were determined by assaying five replicate quality control samples in plasma at four different IFM and IFO concentrations (0.100, 0.250, 5.00, and 50.0 μg/mL and 0.100, 0.500, 50.0, and 100 μg/mL, respectively) in three different analytical runs.

Accuracy was measured as percentage of the nominal concentration. The within-day and between-day precisions were obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as grouping variable.

For the construction of each calibration curve, 8 spiked plasma samples were analyzed in duplicate. After optimization of their weighting factors, linearity of the plasma calibration curves was tested with the *F*-test for lack of fit.

Extraction Recovery

Recovery of extraction of the IFM-derivative was determined by dissolving the isolated IFM-derivative in PPB, resulting in theoretical 1.00 and 10.0

μg/mL solutions, which were diluted further. Full recovery (without sample extraction) was achieved by addition of AcN to the derivative solution in PPB (68:32 v/v), producing a solution identical to the mobile phase, which was injected directly on the HPLC system. Spiked plasma samples were extracted, transferred, evaporated, and, subsequently, reconstituted in mobile phase and injected on the HPLC system, as described above.

Furthermore, 25 mM K_2HPO_4 pH 8.00 was also added to the AcN-layer after extraction (68:32 v/v) of additional samples, producing a solution identical to the mobile phase. By comparing the directly injected IFM-derivative to the extracted samples, with and without the evaporation-step, recoveries were calculated.

Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *p*-values were 0.05 or less.

RESULTS AND DISCUSSION

Chromatography and Detection

The chromatographic method enabled sufficient separation of the analytes. No ion-exchange modifier was used since it did not prove to be essential and better column endurance could be obtained without it. Figure 3 represents typical chromatograms of a blank sample, a patient sample of 3.20 and 14.9 μg/mL IFM and IFO, respectively, and a spiked sample of 5.00 and 25.0 μg/mL IFM and IFO, respectively. The patient's sample was taken at the end of a 1-hour 1.2 g/m² IFO intravenous (i.v.) infusion.

Noteworthy, are the large injection peaks consisting of non-retained DDTC and (derivatized) matrix components, the IFM$(DDTC)_2$ peak at 11.0 min, and the IFO(DDTC) peaks at 29.8 and 31.5 min, respectively. Since the ratio of the exo- and endocyclic IFO derivative peaks was always equal to 5:1 and it can be expected that the molar absorptivities of the compounds are identical, the sum of both peaks was used in the quantitation of IFO.

Sample Pretreatment

Besides stabilization of underivatized IFM,[4] the addition of NaCl in PPB to the plasma sample also prevented mixing of the aqueous and organic-phases

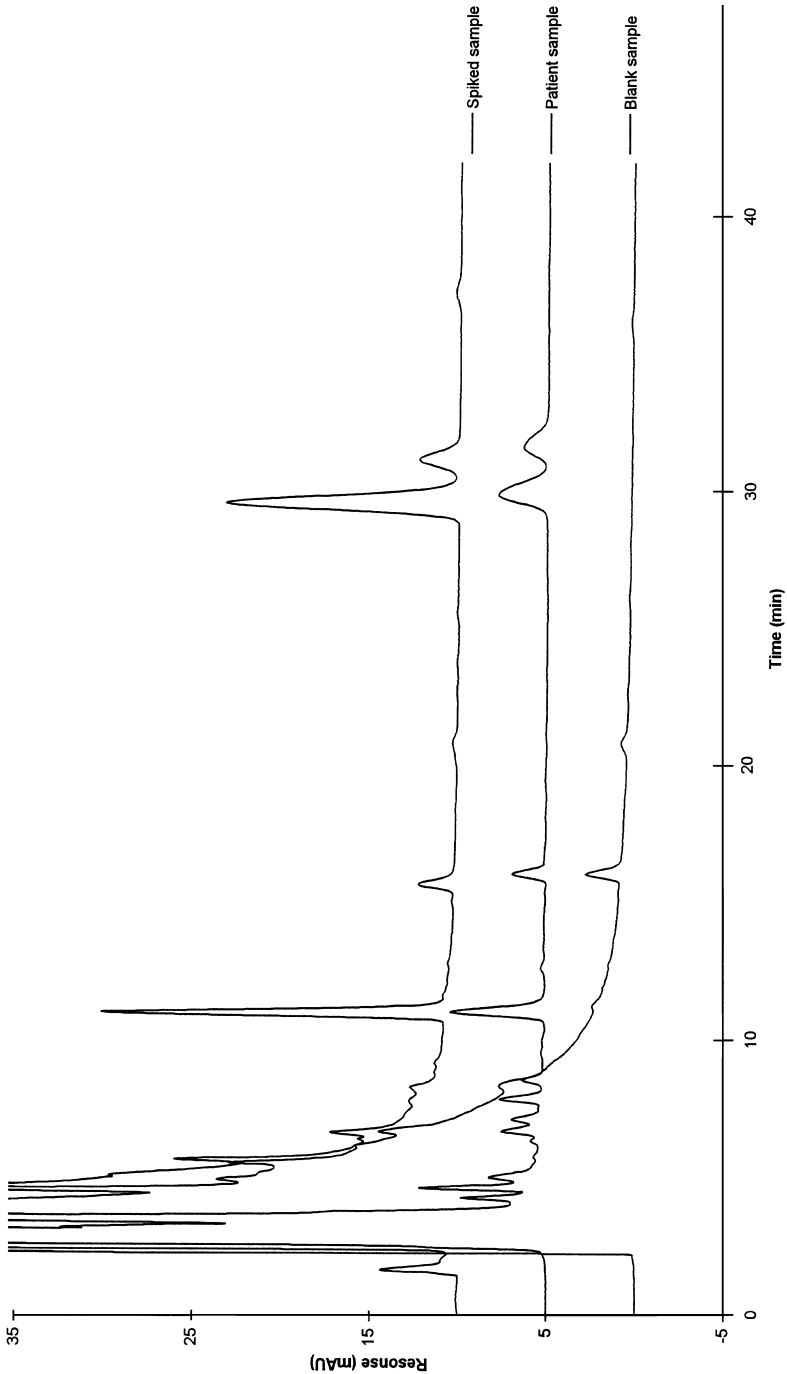

Figure 3. Typical chromatograms of a blank (bottom), a patient (middle) and a spiked (top) plasma sample with 0, 3.20 and 5.00 µg/mL ifosforamide mustard (11.0 min) and 0, 14.9, and 25.0 µg/mL ifosfamide (29.8 and 31.5 min), respectively.

during the liquid-liquid extraction, enabling easy transfer of the organic-layer and preventing undesired co-extraction of water-soluble plasma components. SCZ* addition prevented autocatalytic degradation of 4OHIF. Addition of SCZ* to blank or patient plasma resulted, after a freeze-thaw cycle and subsequent centrifugation, in a clear plasma layer and a pellet. If the plasma was not subjected to this procedure, no reproducible separation of the aqueous and organic-layer could be obtained. The pellet possibly contained plasma components that otherwise prevent a clear separation of the phases.

Identification

The peak of the IFM-derivative was identified as double derivatized IFM(DDTC)$_2$, as depicted in Figure 4. The molecular weight of IFM(DDTC)$_2$ is 446.1, as depicted in Figure 2. Consequently, the parent-peak (MH^+) can be observed at a mass-to-charge ratio (m/z) of 447. The sodium and potassium-adducts were observed at m/z 469 and 485, respectively.

A dimer (M^+_2) was observed at m/z 892 with its typical sodium-adduct at m/z 915. The fragment at m/z 116 and m/z 176 may be explained by $CSN(C_2H_5)_2$ and $C_2H_5SCSN(C_2H_5)_2$ fragments, respectively.

The two IFO peaks were identified as isomeric monomers of IFO(DDTC), as depicted in Figure 2. The DDTC could react with either the chloroethyl-group attached to the exo- or endocyclic nitrogen atom. The molecular weight of IFO(DDTC) is 373.1. Figures 5 and 6 demonstrate similar MS patterns for both isomers. Although differences in relative intensity between fragments were observed, no unique fragments could be distinguished between the two isomers.

The MH^+ was observed at m/z 374 with its typical Cl atom isotope peaks. The sodium and potassium-adducts were observed at m/z 396 and 411. A dimer (M^+_2) was observed at m/z 746 with its sodium and potassium-adducts at m/z 769 and 785. Since, none of the theoretical possible fragments of either the exo- or endocyclic derivatized IFO were observed uniquely with MS, further identification was performed by refragmentation of the parent-peak (MS^2). MS^2 of peak 1 yielded fragments at m/z 92 and 120. These fragments may be explained by $CH_2NHC_2H_4Cl$ and $C_3H_6NHC_2H_4Cl$, respectively.

The exocylic derivative can yield these fragments. The endocyclic derivative cannot. Therefore, it was concluded that IFO peak 1 was the exocyclic derivatized IFO and IFO peak 2 was the endocyclic derivatized IFO.

The UV-absorption spectrum is depicted in Figure 7. A maximum is observed at 276 nm.

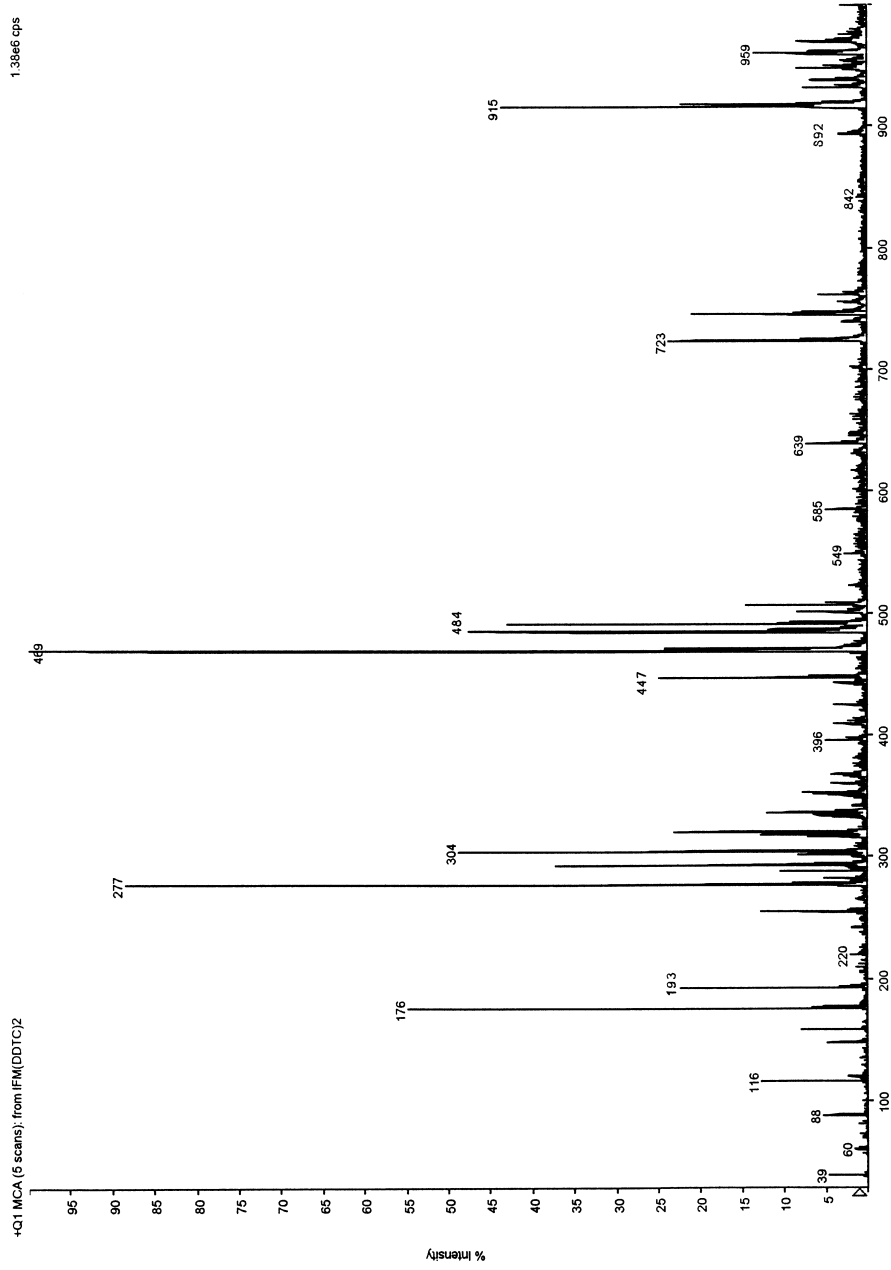

Figure 4. Mass-spectrum of ifosforamide mustard di-derivative (IFM(DDTC)$_2$). IFM(DDTC)$_2$ was isolated at 11.0 min.

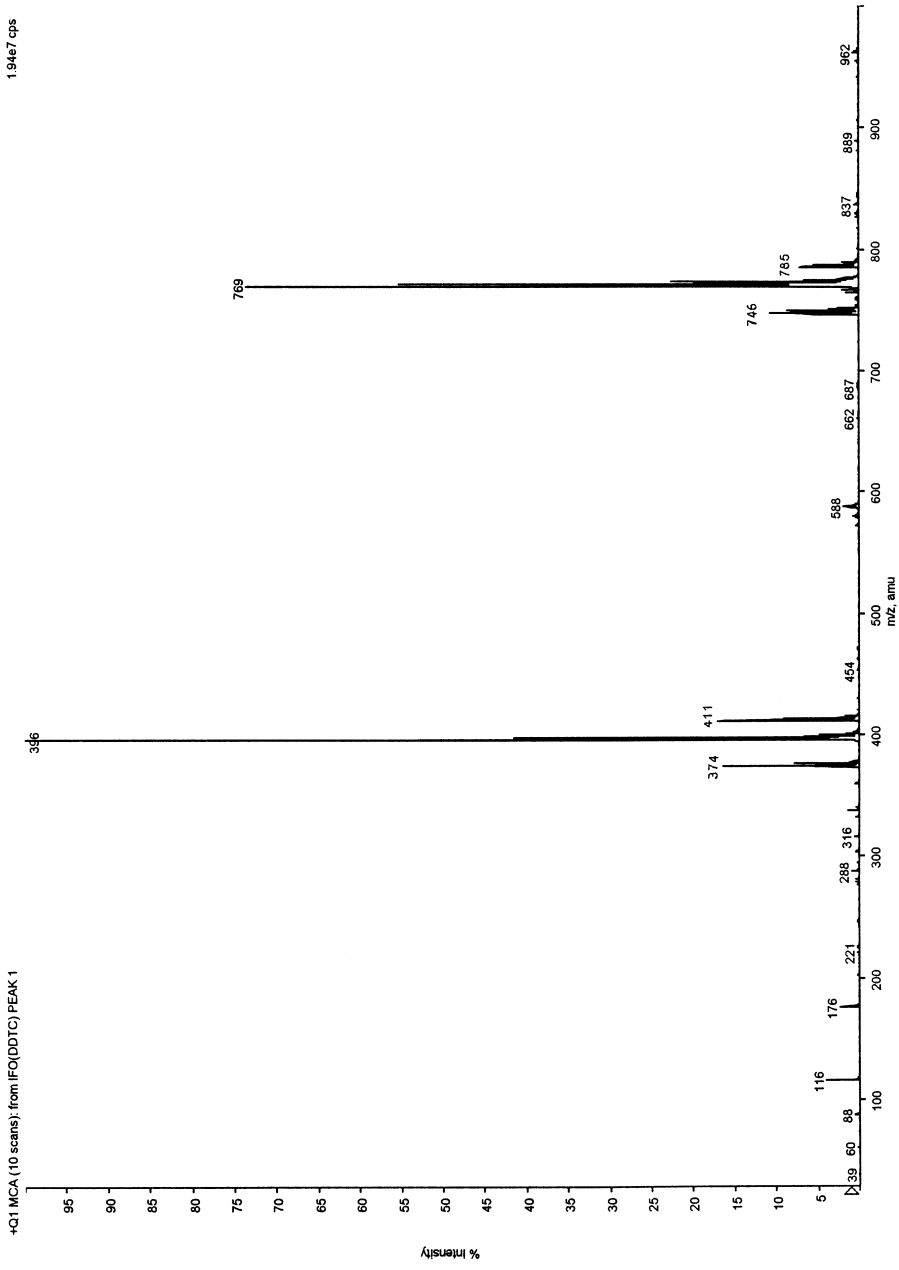

Figure 5. Mass-spectrum of the exocyclic ifosfamide mono-derivative (IFO(DDTC)) isolated at 29.8 min.

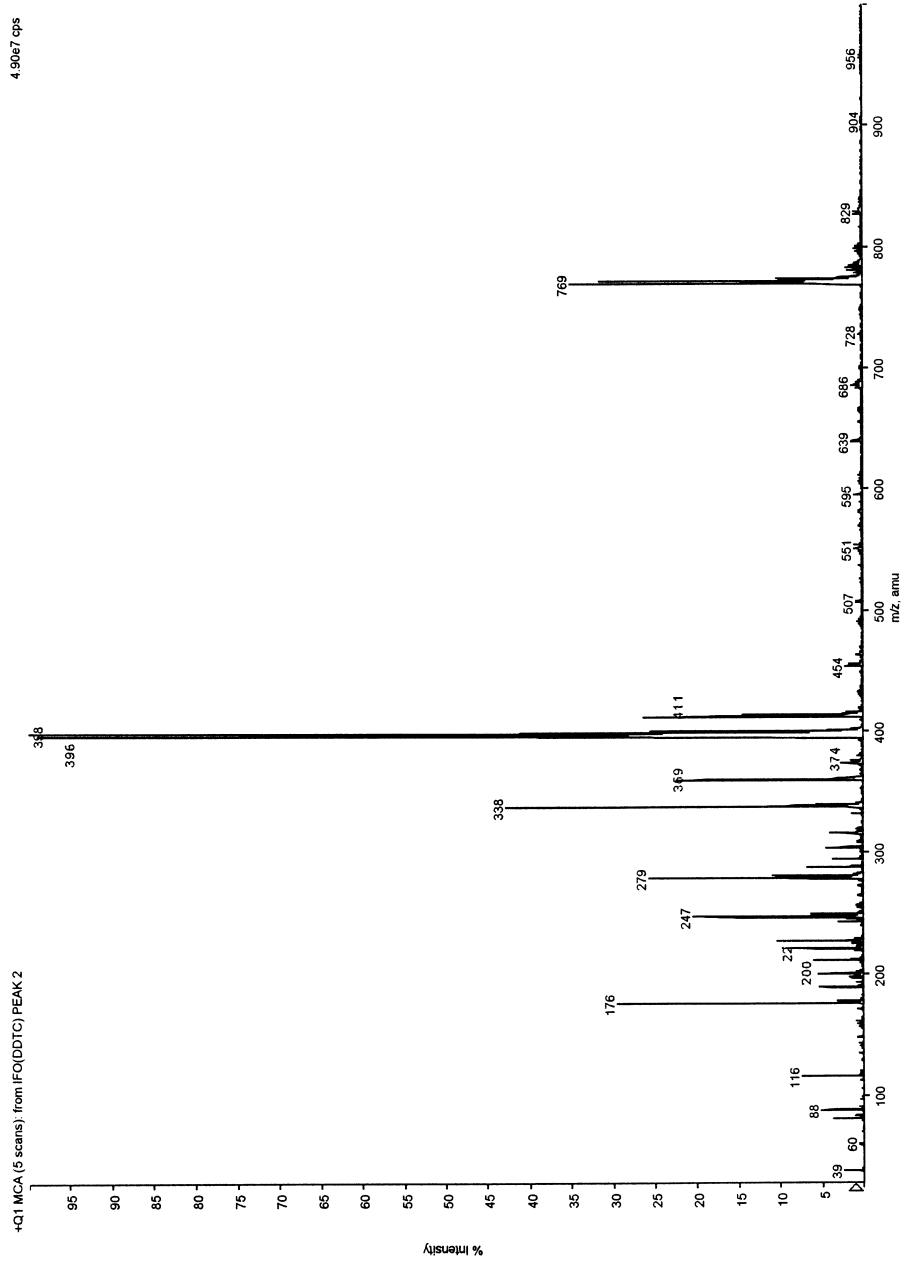

Figure 6. Mass-spectrum of the endocyclic ifosfamide mono-derivative (IFO(DDTC)) isolated at 31.5 min.

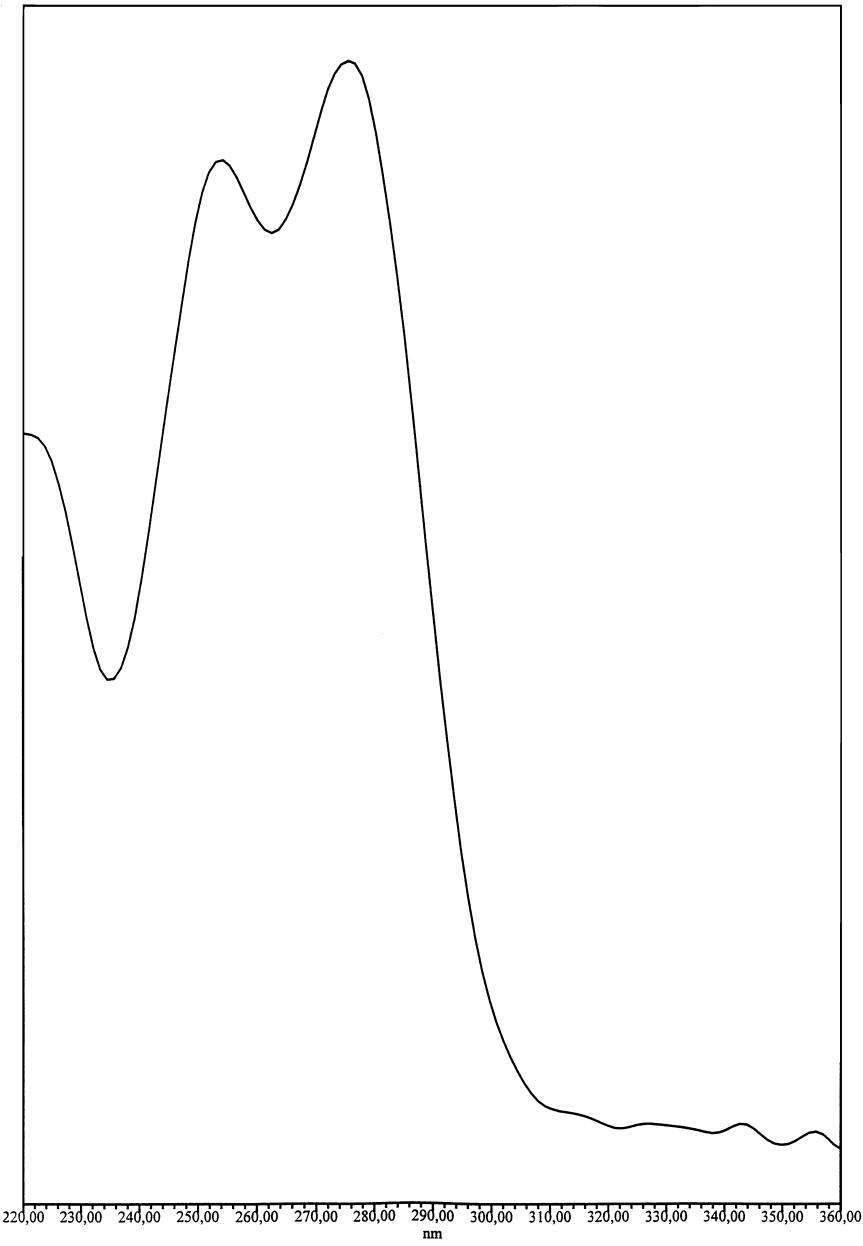

Figure 7. Ultra-violet absorption spectrum of ifosforamide mustard di-derivative.

Specificity and Selectivity

After sample pretreatment, plasma samples spiked with 2-DCEIF, didechloroethylifosfamide, carboxyifosfamide, topotecan, paclitaxel, acetaminophen, caffeine, furosemide, and MESNA did not display any interference with the method.

Non-interfering peaks were detected for 3-DCEIF (9.0 min), 4-ketoifosfamide (19.8 min), ketoconazole (83.5 min), granisetron (6.0 min), dexamethasone (12.5 min), oxazepam (14.5, 21.3 and 26.4 min), temazepam (22.8 min), compared to IFM (11.0 min). Stabilized 4-OHIF (with SCZ) resulted in a peak at 11.4 min. Unstabilized 4-OHIF was spontaneously converted to IFM (11.0 min).

Blank plasma samples of six different individuals showed no interfering endogenous substances in the analysis. Addition of semicarbazide did not interfere with the chromatography.

Optimization of Derivatization

The derivatization temperature did not demonstrate an optimum, but the variability with 0.500 and 5.00 μg/mL IFM at 70°C was less than at 20°C and 40°C. Therefore, 70°C was selected as derivatization temperature. Optimization of derivatization duration scaled to 100% is presented in Figure 8. The optimum derivatization duration was set at 30 min.

The effect of DDTC concentration on the derivatization of IFM is presented in Figure 9. Between 0 and 5.00 mg/mL no IFM derivative could be detected. Between 25.0 and 500 mg/mL DDTC a log-linear relationship between derivative response and DDTC concentration is observed. Although no optimum was reached, chromatographic separation between the unretained DDTC peak and the IFM-derivative deteriorated at reagent concentrations of 500 mg/mL. Therefore, 100 mg/mL DDTC was chosen as the most optimal concentration for derivatization.

Limit of Quantitation

In three analytical runs the LLQs of IFM and IFO were determined in fivefold. The mean percent deviation from the nominal concentration at 0.100 μg/mL IFM and IFO, respectively, were 10.2 and 1.9% with a relative standard deviation of 17.2 and 11.0%, respectively. Therefore, both LLQs were determined to be 0.100 μg/mL.

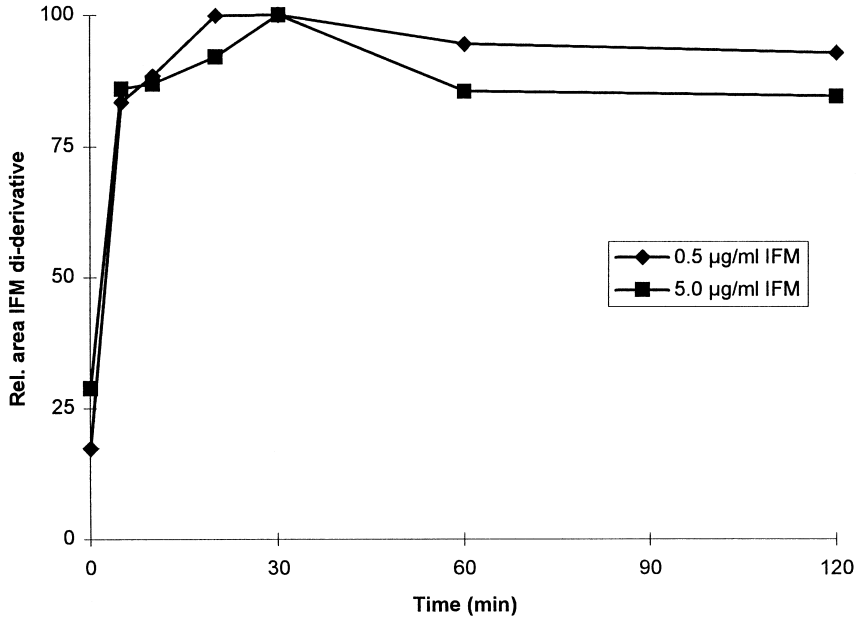

Figure 8. Effect of incubation duration on derivatization of ifosforamide mustard (IFM) with diethyldithiocarbamate.

Validation: Accuracy, Precision, and Linearity

The results from the validation of the method in human plasma are listed in Table 1. The use of the weighting factor of $1/(\text{conc.})^2$ resulted in a minimal sum of squares of residuals from the nominal concentrations. The method proved to be accurate for both analytes (average accuracy at four different concentrations between 89.5 and 110.0% of the nominal concentrations) and precise (within-day precision ranged from 2.8 to 15.8% and between-day precision ranged from 2.0 to 10.0%).

Correlation coefficients (r^2) of calibration curves were always higher than 0.995 as determined by least sum of squares analysis. Calibration curves of IFM and IFO proved to be linear in the range of 0.100-50.0 and 0.100-100 µg/mL, respectively, without lack of fit. Furthermore, no systematic proportional or additive errors were observed.

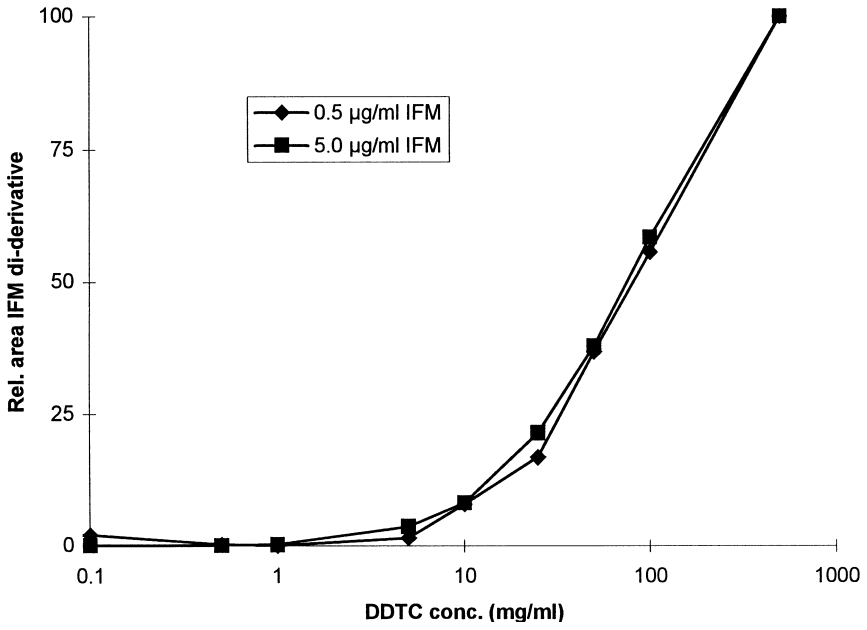

Figure 9. Effect of diethyldithiocarbamate (DDTC) concentration on derivatization of ifosforamide mustard (IFM).

Table 1

Accuracy, Within-Day and Between-Day Precisions of Ifosforamide Mustard (IFM) and Ifosfamide (IFO) Analysis in Human Plasma

Analyte	Conc. (µg/mL)	Accuracy (%) Mean ± S.D.	95% C.I.	Precision (%) Within-Day	Between-Day
IFM	0.100	110.0±17.0	100.6-119.7	15.8	N.A.
IFM	0.250	107.2±15.2	98.7-115.4	11.3	10.0
IFM	5.00	106.6±8.0	102.1-111.0	4.5	7.2
IFM	50.0	101.4±8.3	96.8-106.0	2.8	9.2
IFO	0.100	101.9±11.0	95.8-108.0	11.2	N.A.
IFO	0.500	94.6±5.1	91.8-97.4	5.1	2.0
IFO	50.0	90.6±5.3	87.6-93.5	4.2	4.9
IFO	100	89.5±3.6	87.5-91.5	4.0	N.A.

Abbreviations: S.D. = standard deviation, C.I. = confidence interval, N.A. = not applicable, between-day variation did not exceed within-day variation.

Extraction Recovery

Extraction recoveries (mean ± coefficient of variation) of the IFM-derivative from plasma with the evaporation-step were 62.2 ± 6.7 and 62.4 ± 13.4% for 1.00 and 10.0 μg/mL IFM (n=3), respectively. Extraction recoveries of the IFM-derivative from plasma, without evaporation-step, were 62.9 ± 8.4 and 59.8 ± 3.3% for 1.00 and 10.0 μg/mL IFM spiked sample (n=3), respectively. It is obvious that there is no significant extraction-loss of IFM due to the evaporation-step.

Analysis of Patient Samples

Figure 10 represents a typical pharmacokinetic profile of a patient receiving 3 g/m² IFO i.v. in 3 hours on two consecutive days. IFO and IFM concentrations accumulated over time, but were also eliminated faster on the second

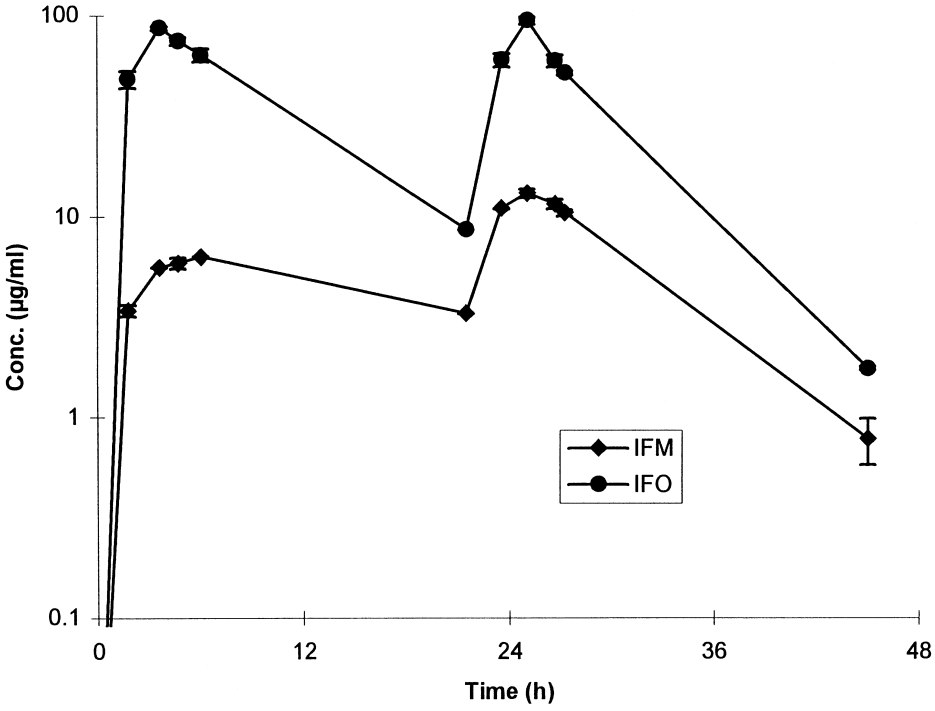

Figure 10. Ifosforamide mustard (IFM) and ifosfamide (IFO) concentrations in plasma in a patient receiving 3 g/m² ifosfamide i.v. in 3 hours on two consecutive days.

day. This resulted in similar maximum IFO concentrations on day 1 and 2. But IFM concentrations were substantially higher on day 2, because more IFO was metabolized to IFM on day 2. This phenomenon can be explained by the auto-induction of IFO and its effect on the metabolism.

CONCLUSIONS

An analytical method for the simultaneous determination of IFM and IFO in human plasma was described. This technique employed derivatization with DDTC followed by deproteinization with AcN. Quantitation was achieved by RP-HPLC with UV-detection. Identification of the derivatized analytes was accomplished using MS and LC-PDA. Incubation settings were optimized. It is our experience that the presented assay can readily be used in a hospital laboratory environment for simultaneous monitoring of IFM and IFO concentrations in patients.

ACKNOWLEDGMENTS

The technical support and assistance in the MS interpretation of R. van Gijn, H. Rosing, and M. Hillebrand is much appreciated.

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Received February 7, 2000 Author's Revisions June 10, 2000
Accepted April 13, 2000 Manuscript 5242