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High-performance liquid chromatographic determination of stabilized 4-hydroxyifosfamide in human plasma and erythrocytes

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Abstract

A method using reversed-phase high-performance liquid chromatography (RP-HPLC) is described for the measurement of the stabilized activated metabolite of ifosfamide, 4-hydroxyifosfamide (4-OHIF), in human plasma and erythrocytes. Immediately after sample collection and plasma–erythrocyte separation at 4°C, 4-OHIF was stabilized by derivatization with semicarbazide (SCZ). The sample pretreatment involved liquid–liquid extraction with ethyl acetate. RP-HPLC was executed with a C_8 column and acetonitrile–0.025 *M* potassium dihydrogenphosphate buffer (pH 7.40)–triethylamine (13.5:86:0.5, v/v) as mobile phase. The analyte was determined with UV detection at 230 nm. Complete validation, optimisation and stability studies were performed and the method proved to be specific, sensitive and with a stable analyte in the range of clinically relevant concentrations (0.1–10 μg/ml) after conventional dosing. The lower limit of quantitation was 100 ng/ml using 1.00 ml of sample. Accuracy was between 94.1 and 107.0%. Within-day and between-day precisions were less than 6.2% and 7.2%, respectively. 4-OHIF-SCZ was found to be stable in the biological matrix at −20°C for at least 1 month. A pharmacokinetic study conducted in a patient receiving 9 g/m^2 over 3 days by means of a continuous infusion, demonstrated the applicability of this method.

Keywords: 4-Hydroxyifosfamide

1. Introduction

Ifosfamide, N,3-bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorine-2-amine 2-oxide (IF, Holoxan, Ifex), is commonly used for the treatment of various solid tumours, soft tissue sarcomas and haematological malignancies in adults and children.

IF is a prodrug which needs activation via the cytochrome *P*450 enzymatic system in the liver to 4-hydroxyifosfamide (4-OHIF). 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. Since 4-OHIF/aldoIF is the activated form of the prodrug IF and because 4-OHIF/aldoIF, in contrast to IFM, is capable of

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penetrating a (tumour) cell, systemic 4-OHIF/aldoIF concentrations may yield the most relevant and easily accessible reflection of a concentration–therapeutic effect correlation. Erythrocytes are of special interest, because it was proposed that they act as transporters of 4-OHIF and 4-hydroxycyclophosphamide (4-OHCP) the activated metabolite of cyclophosphamide, an isomer of ifosfamide [1,2].

Various laborious methods for the determination of 4-OHIF in whole blood, plasma and erythrocytes have been developed. An indirect method widely used is based on the release of acrolein from 4-OHIF, followed by trapping it with 3-aminophenol yielding 7-hydroxyquinoline which is amenable to fluorometric analysis [3,4]. The acrolein that is generated in vivo could interfere with this method, but due to the unstable character of acrolein in blood this is not likely. Another technique applies thin-layer chromatography combined with fluorometric detection [5]. The lower limit of detection with this method is 1 μg/ml and therefore this method is not useful for patient plasma analysis using small volume samples. Furthermore, no stability data were presented. An elaborate method using gas chromatography with mass spectrometric (MS) detection was also developed but its use of self-synthesized deuterium labelled analogues as internal standards renders it less favourable. This method stabilizes 4-OHIF to a cyanohydrin adduct, followed by dichloromethane extraction and silyl derivatization [6]. New methods were developed using ^{31}P nuclear magnetic resonance (NMR) [7] and ^{1}H-NMR-HPLC [8].

Thus far, no relatively simple, reproducible, validated method using HPLC has been developed. The highest hurdle in the analysis of 4-OHIF is its instability in biological fluids. However, by adding semicarbazide (SCZ) the tautomeric equilibrium of 4-OHIF/aldoifosfamide is stabilized with subsequent formation of a stable semicarbazone derivative, as depicted in Fig. 1. This prevents spontaneous decomposition to IFM [9]. An analogous method for the quantitative analysis of 4-OHCP was published recently [10].

Our aim was to develop a novel selective and sensitive HPLC method for 4-OHIF analysis in biological samples, which offers sufficient robustness

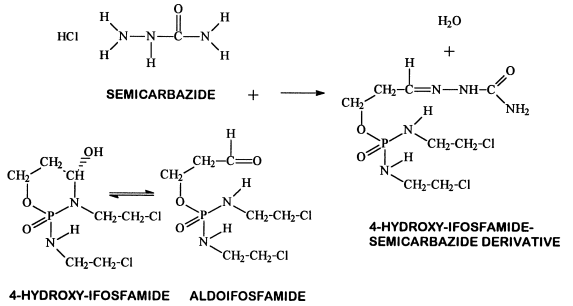

Fig. 1. Stabilization of 4-hydroxyifosfamide by reaction of semicarbazide with the tautomeric aldoifosfamide yielding the derivative 4-hydroxyifosfamide-semicarbazone.

to investigate 4-OHIF pharmacokinetics in patients receiving high dose ifosfamide treatment. The mentioned requirements can be met by stabilizing 4-OHIF with semicarbazide and by using the resulting semicarbazone derivative for HPLC analysis.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Thermo Separation Products (TSP) Spectraseries System with a model AS3000 automated injector with a 100-μl loop and a model P1000 pump connected to a model UV1000 detector with ultra-violet detection at 230 nm. Data were collected by the TSP system and transferred by model SN4000 analog–digital convertor to a Dell optiplex Gs Pentium personal computer. A 5-μm Merck Hibar LiChrosorb C_8 RP-Select B (25 cm×4.0 mm I.D.) column was equipped with a 10-μm Chrompack precolumn.

2.2. Chemicals

4-Hydroperoxyifosfamide (4-OOH IF) (Lot 033.6496) and all other ifosfamide and cyclophosphamide metabolites were a kind gift of Dr. J. Pohl, Asta Medica (Frankfurt, Germany). Acetonitrile and ethanol (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Ethyl acetate (analytical reagent grade), triethylamine (p.s. grade) and anhydrous potassium dihydrogenphosphate (suprapure grade) were obtained from Merck

(Darmstadt, Germany). Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). A 2 *M* SCZ solution was made and adjusted to pH 7.40 with 1 *M* HCl and 1 *M* NaOH, prior to use. Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands). Blank erythrocytes were collected from volunteers in the Slotervaart Hospital (Amsterdam, The Netherlands).

2.3. Preparation of standards

4-OOH IF was used as a substitute for 4-OHIF. In aqueous solution, 4-OOH IF rapidly liberates 4-OHIF/aldoifosfamide and hydrogen peroxide. Fresh solutions of 4-OHIF were prepared immediately prior to use by dissolving an equimolar amount of 4-OOH IF in distilled water. Dissolution was accelerated by ultra-sonication. Standard concentration curves were obtained by adding 1 ml drug-free plasma of 4°C to known amounts of unstabilized 4-OHIF in the range 0, 0.10, 0.25, 0.50, 1.0, 5.0 and 10.0 μg/ml in 5-ml polypropylene reaction tubes. Immediately after vortexing for 15 s, 100 μl 2 *M* SCZ (pH 7.40) were added and vortexed rigorously for 1 min.

2.4. Patient sample collection

Venous blood samples of a patient receiving intravenous ifosfamide treatment (9 g/m^2/72 h) were taken at 0, 3, 10, 20, 24, 48, 68, 72, 73, 76, 80 h after the start of treatment. The samples were collected in 5-ml polypropylene reaction tubes and immediately cooled by placing them in ice–water. The samples were then centrifuged without delay for 5 min with 1000 *g* at 4°C. Two 1-ml plasma samples were collected and added to 100 μl 2 *M* SCZ (pH 7.40). The left-over plasma was collected for ifosfamide analysis according to the method as described by Kaijser et al. [11]. The white blood cell layer was discarded with sufficient margins. A 1-ml volume of erythrocytes was collected from the erythrocyte-layer and added to 100 μl 2 *M* SCZ (pH 7.40). The left-over erythrocytes were also collected for ifosfamide analysis. All SCZ-stabilized samples were vortexed rigorously for 1 min and stored at −70°C pending analysis. The sample collection and stabilization procedure is performed within 10 min.

2.5. Sample pretreatment

After thawing, 3 ml ethyl acetate was added to all patient and standard samples. After vortexing for 1 min the samples were centrifuged for 10 min with 1000 *g*. The organic layer was separated and evaporated to dryness under a gentle nitrogen flow at 40°C. The samples were reconstituted in 150 μl mobile phase. Total reconstitution was achieved by ultrasonicating the sample for 15 min followed by vortexing for 1 min. The injected volume was 100 μl.

2.6. Chromatography

The mobile phase consisted of acetonitrile–0.025 *M* potassium dihydrogenphosphate buffer–triethylamine (13.5:86:0.5, v/v) adjusted to pH 7.40 with 2 *M* hydrochloric acid. The UV wavelength for detection was 230 nm. The flow-rate was 0.8 ml/min. The analytical column was operated at ambient temperature.

2.7. Mass spectrometric identification of 4-OHIF-SCZ

A solution containing 5.7 mg 4-OHIF was derivatized with 9.2 mg SCZ (an approximately four times equimolar SCZ amount). After extraction with ethyl acetate the derivative was evaporated to dryness. Also, a 1-ml volume of eluted 4-OHIF-SCZ was collected after HPLC analysis and extracted with ethyl acetate. The residues were reconstituted in a 50% acetonitrile solution and injected in an electron spray VG Platform (Fisons instrument) electron impact MS system for identification.

2.8. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank plasma and erythrocytes samples. The following related compounds were investigated for interference with the analytical method: ifosfamide, 4-ketoifos-

famide, carboxyifosfamide, 2-dechloroethylifosfamide, 3- dechloroethylifosfamide, ifosforamide mustard and 4-OHCP-SCZ. Possible comedication was also tested for interference. The substances tested were granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, topotecan and sodium 2-mercaptoethanesulphonate (MESNA).

2.9. Limit of quantification and limit of detection

The lower limit of quantitation (LLQ) was 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 limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The upper limit of quantitation (ULQ) was arbitrarily defined as 10 μg/ml.

2.10. Validation: accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precisions of the method were determined by assaying six replicate standard samples in plasma at four different 4-OHIF concentrations (0.1, 0.5, 5 and 10 μg/ml) in three different analytical runs. These quality control samples were made independent of standard curve concentrations. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and between-day precision were obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as group variable. For the construction of each calibration curve seven spiked plasma samples were analyzed in duplicate. After optimisation of their weight factors, linearity of the three plasma and three erythrocyte calibration curves was tested with the *F*-test for lack of fit. Recovery of 4-OHIF-SCZ after the sample pretreatment procedure was determined by comparing observed 4-OHIF-SCZ in extracted plasma and erythrocytes samples to those of nonprocessed standard solutions made of 4-OHIF-SCZ diluted in mobile phase and direct injection.

2.11. Optimisation

The possible breakdown of 4-OHIF before stabilization with SCZ was determined. This was carried out by exposing standard plasma samples of 0.5 and 5 μg/ml 4-OHIF in duplicate, unprotected by SCZ, to 4°C, room temperature and 37°C for 0, 10, 20, 30, 60 and 90 min. Directly after exposure the standards were stabilized with SCZ and stored at −20°C pending standard analysis. Optimisation of incubation was tested by using standard plasma samples of 0.5 and 5 μg/ml 4-OHIF in triplicate, stabilized with SCZ and exposing them to 4°C, room temperature, 40°C and 60°C for 0, 10, 20 and 60 min. The influence of the amount of SCZ on the stability and chromatography was tested by adding various amounts of SCZ (100 μl of 0.125, 0.25, 0.5, 1.0 and 2.0 *M*) to standard plasma samples of 0.5 and 5 μg/ml 4-OHIF in duplicate, followed by sample pretreatment as described.

2.12. Stability during storage

Stability during storage was tested by storing standard plasma samples of 0.5 and 5 μg/ml 4-OHIF in triplicate, stabilized with SCZ, at 37 and 60°C for 1 and 24 h, at room temperature for 1 h, 24 h, 3 days and 7 days, and at −20 and −70°C (including three additional freeze–thawing cycles) for 1 h, 24 h, 3 days, 7 days and 1 month. After the storage period the samples were treated as described and after organic layer evaporation stored as dry samples at −70°C, pending further analysis. Analysis was always done within 1 month.

2.13. 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.

3. Results and discussion

3.1. Chromatography and detection

A new bioanalytical assay has been developed for the determination of 4-OHIF. Recently, a bioanalytical assay was described for 4-OHCP [10]. Although some parallels exist between the two methods, the chromatography was substantially changed. Furthermore, a detailed optimisation and stability study was performed in order to validate every step in the handling of the samples from collection in the clinic to bioanalysis in the laboratory. The spectral analysis of 4-OHIF-SCZ displayed a maximal chromophoric activity at 230 nm. The reversed-phase chromatography was investigated using different mixtures of acetonitrile in order to find the optimal combination of good separation and a practicable retention time for the analyte. By raising the pH to 7.40 full separation of interfering peaks and the analyte was achieved. Peak shape was further optimized by addition of 0.5% triethylamine.

3.2. Sample pretreatment

For 4-OHCP-SCZ a two-step liquid–liquid extraction (LLE) with ethyl acetate–chloroform (3:1, v/v) was described in order to separate the analyte from interfering substances [10]. Since no interfering peaks were found for 4-OHIF-SCZ with the chromatography as described above, extraction could be reduced to a single step. Furthermore, the extraction efficacy could be increased by using ethyl acetate alone. Optimal balance between extraction of 4-OHIF-SCZ and efficient removal of interfering endogenous compounds was found to be at pH 7.4. Alkalinization to pH 12 resulted in a higher extraction efficacy, but this coincided with increased levels of interfering substances. Since, 4-OHCP-SCZ is also detectable its usefulness as an internal standard was investigated. However, 4-OHCP is not commercially available, thus rendering it a less preferable internal standard. In addition, accuracy, within-day and between-day precisions, without an internal standard, were within accepted limits, thus an internal standard was not considered essential. By adding 100 μl SCZ of pH 7.40 to 1 ml plasma at an approximate pH of 7.4, sufficient buffering capacity during LLE was accomplished. LLE of 1 ml plasma resulted in turbid reconstituted samples. However, by ultra-sonicating, clear solutions were obtained with no chromatographic complications when 100 μl of the 150 μl reconstituted sample were injected.

3.3. Mass spectrometric identification of 4-OHIF-SCZ

The directly derivatized preparation and the eluted preparation of 4-OHIF-SCZ were both tested on the HPLC system as described in this paper, before MS analysis. No evidence of impurity could be detected with HPLC, but the eluted preparation contained relatively high amounts of various salts originating from the mobile phase, which interfered with the MS analysis. The mass spectrogram of the preparative sample of 4-OHIF-SCZ is shown in Fig. 2. At increasing ionization strengths more fragmentation was observed, but all spectra contained the parent peak with m/z of 334.1 (M^+) with its characteristic chlorine isotopes. The sodium adduct at m/z 356 ($M^+ + Na$) and the potassium adduct at m/z 374 ($M^+ + K$) confirmed the parent peak. Furthermore, a dimer (M_2^+) and trimer (M_3^+) with similar fragmentation patterns were observed, albeit at 10 and 100 times lower intensities. The major fragmentation peaks were at m/z 80 ($NH_3^+CH_2CH_2Cl$), 249 ($M^+ - CNNHCONH_2$), 259 ($M^+ - NH_2NHCONH_2$), 275 ($M^+ - NHCONH_2$). The peak at m/z 293 ($M^+ - NHCONH_2 + H_2O$) could be explained by addition of water (at the C-atom bonded to the N-atom of SCZ) and subsequent fragmentation. The characteristic chlorine isotope fragmentation pattern suggested preservation of both chloroethyl-groups. Subsequently, the peak at m/z 315 ($M^+ + Na\text{-}NHCN$) was the sodium adduct of the rearranged fragment.

3.4. Specificity and selectivity

After normal sample pretreatment with SCZ and LLE, plasma samples spiked with ifosfamide, carboxyifosfamide, 2-dechloroethylifosfamide, 3-dechloroethylifosfamide, ifosforamide mustard,

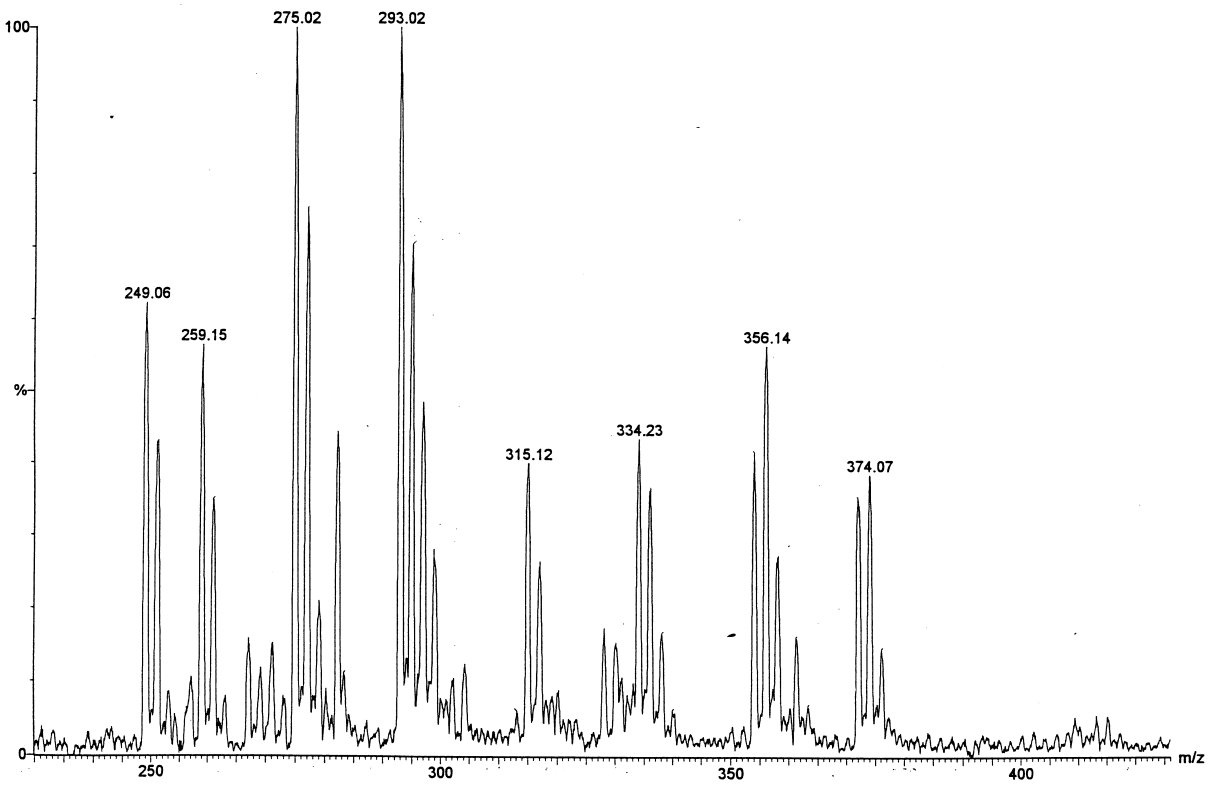

Fig. 2. Mass spectrogram of the derivative 4-hydroxyifosfamide-semicarbazone (M^+, m/z 334) by means of electron spray electron impact mass spectrometry.

granisetron, dexamethasone, acetaminophen, oxazepam, temazepam, topotecan and MESNA did not display any interference with the chromatography. Blank plasma and erythrocytes samples of six different individuals showed no interfering endogenous substances in the analysis of 4-OHIF-SCZ. Typical chromatograms of blank erythrocyte and plasma samples and erythrocyte and plasma patient samples of 1.0 μg/ml are shown in Fig. 3. However, the SCZ derivative of 4-ketoifosfamide did appear in the chromatogram (t_R 16.5 min), well separated of 4-OHIF-SCZ (t_R 13.0 min). Limited analysis of 4-ketoifosfamide revealed that the lower limit of quantification was approximately 5 μg/ml from a 1-ml plasma sample. Furthermore, 4-OHCP-SCZ (t_R 15.5 min) can be detected, well separated of 4-OHIF-SCZ.

3.5. Limit of quantification and limit of detection

In three analytical runs the LLQ was determined in five-fold. The percent deviation from the nominal concentration (100 ng/ml) and the relative standard deviation (R.S.D.) were less than 18.1% and 7.5%, respectively. Thus, 100 ng/ml was defined to be the LLQ. The LOD in plasma was 10 ng/ml, with a signal-to-noise ratio of 3.

3.6. Validation: accuracy, precision, linearity and recovery

The results from the validation of the method in human plasma are listed in Table 1. The use of the weight factor of $1/(\text{conc.})^2$ resulted in a minimal sum of deviations from nominal concentrations. The

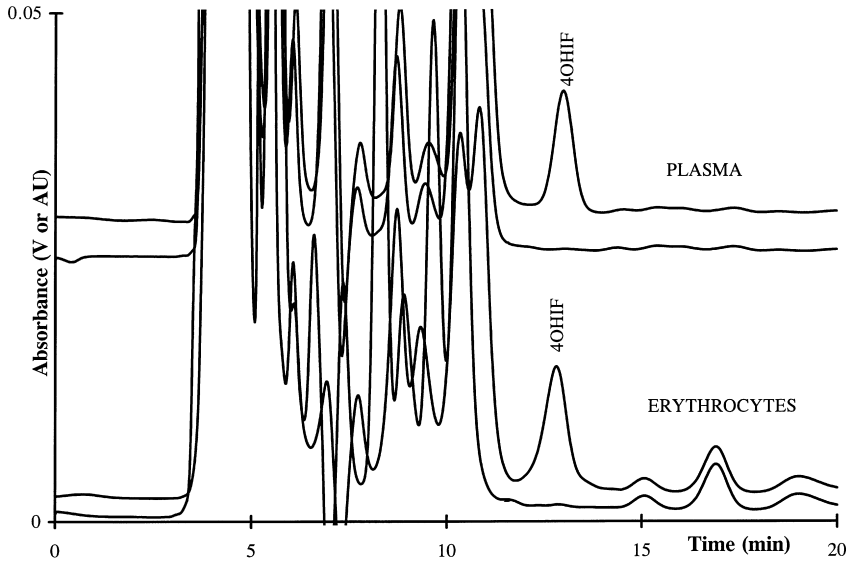

Fig. 3. Chromatograms of a 1-ml blank erythrocyte sample (lower curve of lower set of curves), a 1-ml patient erythrocyte sample containing 1.0 μg/ml 4-hydroxyifosfamide (upper curve of lower set of curves), a 1-ml blank plasma sample (lower curve of upper set of curves) and a 1-ml patient plasma sample containing 1.0 μg/ml 4-hydroxyifosfamide (upper curve of upper set of curves).

method proved to be accurate (average accuracy at four different concentrations between 94.1 and 107.0% of the real concentrations) and precise (within-day precision ranged from 2.2 to 6.8% and between-day precision ranged from 2.6 to 7.2%). Correlation coefficients (r^2) of calibration curves were always higher than 0.995 as determined by least squares analysis. Calibration curves proved to be linear in the range 0.1–10 μg/ml with use of the F-test for lack of fit as an indicator of linearity of the regression model. Extraction recoveries from plasma were 96.0±11.2 and 99.8±3.7% for a 0.5 and 1.0 μg/ml 4-OHIF spiked sample (n=3), respectively. Similar, 4-OHIF was extracted from erythrocytes with 108.6±9.5 and 118.8±4.3% recovery, respectively. Since extraction recovery of erythrocytes were equal to that of plasma, a limited validation of the analysis in erythrocytes is sufficient.

3.7. Optimisation

Degradation during sample collection of underivatized 4-OHIF determined under various conditions is

Table 1
Accuracy, within-day and between-day precision of the analysis of the 4-hydroxyifosfamide- semicarbazone derivative in human plasma[a]

Concentration (μg/ml)	Accuracy (%)		Precision (%)		
	Mean±S.D.	95% C.I.	Mean±S.D.	Within-day	Between-day
0.1	94.1±7.3	90.1–98.2	94.4±7.9	6.0	6.8
0.5	100.0±10.3	94.3–105.7	110.4±8.8	6.2	7.2
5.0	107.0±4.2	104.7–109.3	108.3±3.4	2.2	2.6
10.0	101.8±4.1	99.6–104.1	102.6±4.0	2.3	3.7

[a] Calculated using one-way ANOVA (P<0.05). Total number of different runs, total number of replicates per run for accuracy and total number of replicates per run for precision were 3, 5 and 4, respectively.
S.D.=standard deviation, C.I.=confidence interval.

Table 2
Effect of temperature and exposure time on degradation of 4-hydroxyifosfamide without semicarbazide protection in human plasma during sample collection

Temperature (°C)	Concentration (μg/ml)	Degradation (% nominal conc.)±C.V. (%)					*n*
		10 min	20 min	30 min	60 min	90 min	
4	0.5	100.0±0.8	94.5±4.1	76.7±0.3	85.2±1.5	87.9±4.6	3
	5.0	100.0±2.7	94.4±1.7	93.8±3.0	93.0±0.5	83.0±0.6	3
25	0.5	98.0±1.5	94.3±1.6	75.7±3.6	57.9±2.0	56.5±2.4	3
	5.0	87.2±0.8	76.4±4.3	64.3±7.2	40.0±2.8	23.3±15.0	3
37	0.5	69.9±6.7	64.3±3.1	40.6±1.3	44.2±8.6	28.1±6.4	3
	5.0	67.0±4.6	39.8±4.9	18.1±13.4	9.9±6.5	7.8±14.9	3

n=Total number of replicates; C.V.=coefficient of variation.

shown in Table 2. With higher temperature and increasing time, breakdown is observed at both concentrations. Most relevant is the observation that at 4°C during the first 20 min 4-OHIF is stable. The sample collection process in the clinic was always at 4°C and within 10 min, starting directly after collecting the blood until SCZ derivatization and storage.

Optimal incubation during derivatization of 4-OHIF with SCZ is shown in Table 3. Belfayol et al. reported optimal incubation for the formation of 4-OHCP-SCZ at 60°C for 60 min [10]. Our investigations on the formation of 4-OHIF-SCZ show an optimal incubation at 4°C with minimal exposure. At 60°C for 1 h a 60% reduction of 4-OHIF-SCZ can be observed. Although the reaction of 4-OHIF with SCZ might be maximized at higher temperatures, the still underivatized 4-OHIF is susceptible to breakdown under noncooled conditions. Therefore, the best method of derivatization appears to be a fast and cooled incubation with SCZ. This guarantees full, albeit slow, derivatization, but without unwanted degradation of 4-OHIF. Alteration of the amount of added SCZ (0.125–2 *M*) did not influence the intensity of the 4-OHIF-SCZ or the noninterfering endogenous peaks.

3.8. Stability during storage

The stability during storage of 4-OHIF-SCZ in biological matrix is represented in Table 4. During the first month the degradation of 4-OHIF-SCZ in biological matrix was less than 3% at −70°C and 10.6% at −20°C. Stored pretreated 4-OHIF-SCZ samples (dry residues after LLE and solvent evaporation) were also stable for at least 1 month. By storing biological samples containing 4-OHIF-SCZ at most

Table 3
Effect of temperature and incubation duration on derivatization of 4-hydroxyifosfamide with semicarbazide in human plasma during sample pretreatment

Temperature (°C)	Concentration (μg/ml)	Recovery (%)±C.V. (%)			*n*
		10 min	20 min	60 min	
4	0.5	100.0±20.2	82.7±2.1	73.3±0.4	2
	5.0	77.7±6.3	100.0±11.4	89.1±1.2	2
25	0.5	65.0±0.4	51.0±10.7	40.4±5.5	2
	5.0	92.5±7.1	79.5±2.5	87.1±9.8	2
40	0.5	17.9±1.0	18.6±6.1	18.1±2.0	2
	5.0	52.3±4.1	46.1	47.2±1.2	2
60	0.5	22.1±3.4	15.9±9.0	19.6±0.7	2
	5.0	51.1±0.8	41.1±3.7	39.2±6.8	2

n=Total number of replicates; C.V.=coefficient of variation.

Table 4
Stability during storage of human plasma samples spiked with 4-hydroxyifosfamide-semicarbazone derivative

Storage temp. (°C)	Concentration (μg/ml)	Recovery (%)±C.V. (%)					*n*
		1 h	24 h	3 days	7 days	1 month	
60	0.5	100±1.7	52.6±1.4	N.d.	N.d.	N.d.	3
	5.0	100±0.5	47.4±0.7	N.d.	N.d.	N.d.	3
37	0.5	100±2.0	81.0±0.5	N.d.	N.d.	N.d.	3
	5.0	100±2.9	84.2±1.0	N.d.	N.d.	N.d.	3
25	0.5	100±1.3	91.9±3.9	74.4±1.4	73.0±4.8	N.d.	3
	5.0	100±2.4	89.7±0.7	79.5±3.1	77.6±1.5	N.d.	3
−20[a]	0.5	100.0±5.5	N.d.	105.5±4.1	101.8±5.5	100.6±3.1	3
	5.0	100.0±2.6	N.d.	104.9±0.8	93.2±2.3	89.4±1.7	3
−70[a]	0.5	100.0±6.6	N.d.	100.1±5.9	100.3±5.8	97.0±5.2	3
	5.0	100.0±2.2	N.d.	104.0±1.6	100.8±2.5	104.5±0.5	3

n=Total number of replicates; C.V.=coefficient of variation; N.d.=not determined.
[a] Including three freeze–thaw cycles.

for 1 month at −20°C before analysis sample stability is not jeopardized.

3.9. Analysis of patient samples

The applicability of the 4-OHIF assay for pharmacokinetic studies in patients receiving ifosfamide treatment was demonstrated, as depicted in Fig. 4. The assay allowed monitoring of plasma and erythrocytes levels of 4-OHIF. In addition, the erythrocytes can be used to investigate preferential transport routes of 4-OHIF throughout the body [1]. Since, 4-OHIF is the transport form directly prior in the cascade to the ultimate active metabolite ifosforamide mustard, and the latter is unable to penetrate cell walls, it is the most relevant metabolite in blood for monitoring ifosfamide pharmacokinetics and metabolism in relation to efficacy of ifosfamide treatment.

4. Conclusions

A validated assay for the quantitative determination of 4-OHIF in human plasma and erythrocytes is described. The assay meets the current require-

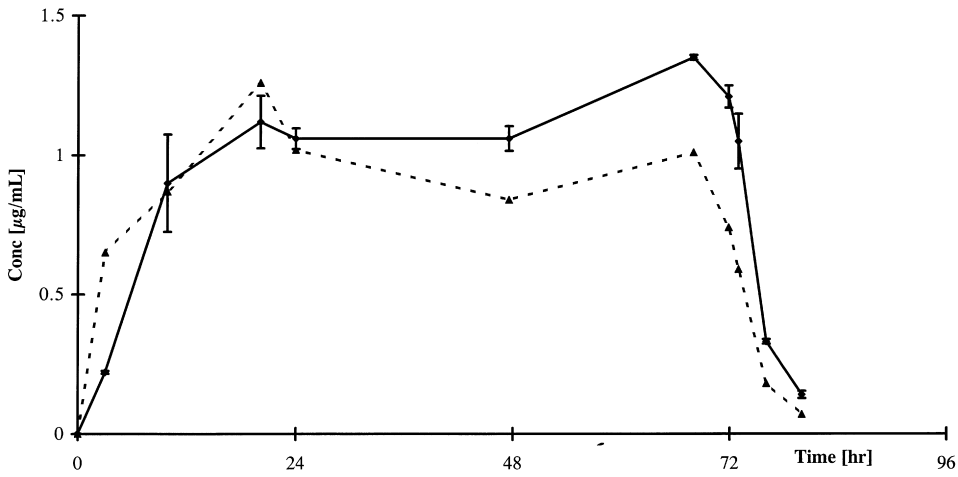

Fig. 4. 4-Hydroxyifosfamide concentrations in plasma and erythrocytes (solid and dashed line, respectively) in a patient receiving an intravenous infusion of 9 g/m^2 ifosfamide over 72 h.

ments for the validation of a bioanalytical method and can be used for pharmacokinetic studies in ifosfamide treated patients. It is our experience that the currently described HPLC assay can readily be used in a hospital laboratory environment for monitoring of 4-OHIF concentrations.

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