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Chromatographic analysis of the enantiomers of ifosfamide and some of its metabolites in plasma and urine

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Abstract

The enantiomers of the cytostatic drug ifosfamide and the two metabolites 2- and 3-dechloroethylifosfamide were isolated from plasma and urine by liquid–liquid extraction with ethyl acetate, resolved on a Chirasil-L-val gas chromatographic column and detected by a nitrogen–phosphorus-selective flame ionisation detector. Resolution of the racemic compounds for identification purposes was also accomplished with high-performance liquid chromatography on a chiral column. The validated gas chromatographic method was suitable to determine the total concentrations and the enantiomeric composition of ifosfamide and its dechloroethylated metabolites in plasma and urine samples from treated patients. Some metabolic preferences in the metabolism of ifosfamide were found.

Keywords: Enantiomer separation; Ifosfamide; Dechloroethylifosfamine

1. Introduction

The oxazaphosphorine drug ifosfamide (IF) needs biotransformation in order to develop its cytotoxic effect. The activation pathways leads via 4-hydroxylation to ifosforamide mustard, a strong alkylating compound. However, oxidation of the side chain on the exo- or endocyclic nitrogen of IF leads to the dechloroethylated metabolites, 2- and 3-dechloroethylifosfamide (2- and 3-dechloroethylIF), respectively, which are devoid of cytotoxic activity.

The IF molecule contains an asymmetrically substituted phosphorous atom and exists in two enantiomeric forms, (−)-(*S*)-ifosfamide and (+)-(*R*)-ifosfamide (*S*-IF and *R*-IF, respectively, Fig. 1). In current clinical practice, IF is administered as a racemic mixture (*rac*-IF). Previous investigations have revealed a considerable variability in metabolism and cytotoxicity of the enantiomers of oxazaphosphorines among different animal species and tumor models [1–8]. Despite the indications for biological differences between the enantiomers of IF, the majority of the reported pharmacological studies of IF have been performed with racemic IF and were evaluated without any consideration for the pharmacokinetic and metabolic fate of the enantiomers [9–14]. This was mainly due to the lack of a sensitive method of analysis of the enantiomers of IF and its metabolites which could be used for routine pharmacokinetic analysis.

Chiral gas chromatography (GC) or high-performance liquid chromatography (HPLC) has been used

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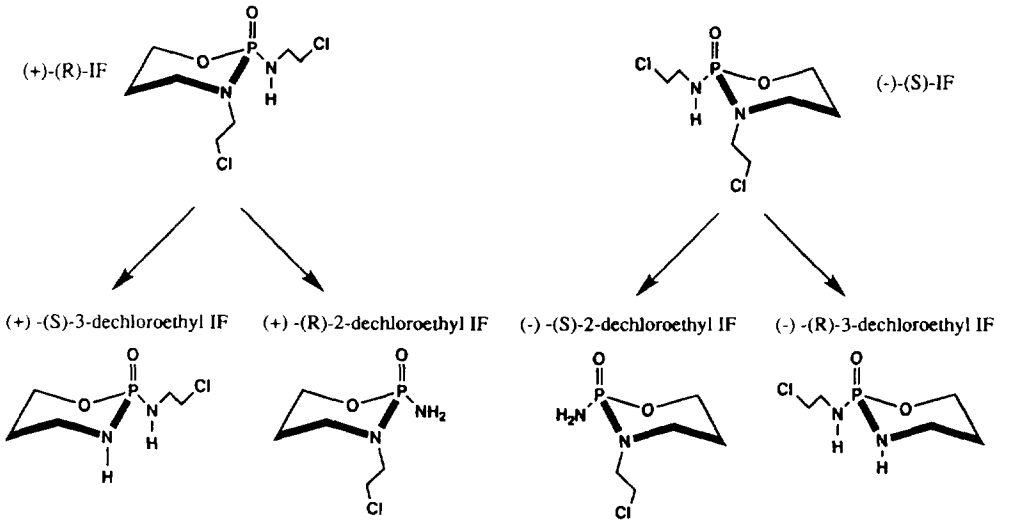

Fig. 1. Molecular structures of the enantiomers of IF and 2- and 3-dechloroethylIF.

for the determination of the enantiomers of IF. In the studies of Blaschke et al. [15–17] only the urinary concentrations of the enantiomers of IF and the dechloroethylated metabolites were determined using an enantioselective gas chromatographic (GC) assay. The method was not validated and the analysis time was more than 40 min, resulting in peak broadening and loss of sensitivity. Recently, another enantioselective GC method has been published by Young et al. [18]. The IF enantiomers were separated from the plasma matrix by solid phase extraction (SPE), resolved by GC on a chiral stationary phase and detected by mass quantitative selected ion monitoring. The assay has been validated for routine clinical and pharmacokinetical use. However, the analysis of 2- and 3-dechloroethylIF was not included. Although complete chromatographic resolution of the two IF enantiomers was not achieved, the method was able to resolve and quantify samples accurately with a limit of detection of 250 ng IF-isomer per ml plasma. A method, developed by Granville et al. [19] using a stereoselective, cyclodextrin-based GC column, gave satisfying results. However, the method was not very suitable for routine analysis, due to the employment of a non-commercially available column and a mass detector. Moreover the detection limit was not very satisfactory.

The enantiomers of IF have also been resolved using enantioselective HPLC [20–23]; however, for bio-analytical research these assays are too laborious and lack the desired sensitivity. Therefore, a simple and sensitive GC method has been developed in order to study the pharmacokinetic disposition of the enantiomers of IF in plasma and urine of cancer patients. With this method the total concentration as well as the enantiomeric composition of IF, 2- and 3-dechloroethylIF can be determined in one single run.

2. Experimental

2.1. Chemicals

IF, trofosfamide (TF), 2- and 3-dechloroethylIF were kindly donated by Asta Medica, (Frankfurt, Germany). All other chemicals were of analytical grade and were used as received. Throughout the study filtered demineralised water was used (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The chiral HPLC system, based on the work of Masurel and Wainer [20], consisted of a Model 6000A solvent delivery system, a U6K injection device (both from Waters Assoc., Milford, MA,

USA) and a Spectroflow 773, variable-wavelength detector (ABI Analytical, Kratos Division, Ramsey, NJ, USA) operating at 205 nm. Separation was achieved on a Chiralcel OD column, 250×0.46 cm I.D. (J.T. Baker, Phillipsburg, NJ, USA) with *n*-hexane–2-propanol (80:20 v/v) as the mobile phase at a flow-rate of 0.5 ml/min with the column at ambient temperature. Quantitation of IF, 2- and 3-dechloroethylIF enantiomers was based on peak-area measurements using a SP4270 integrator (Spectra Physics, Santa Clara, CA, USA).

The micropolarimeter used for the identification of eluted peaks was a Jasco DIP-4 Digital Polarimeter (Tokyo, Japan).

Chiral gas chromatography, of IF, 2- and 3-dechloroethylIF was performed similarly to Young al. [18], using a Carlo Erba HRGC 5300 (Milan, Italy) equipped with a Chirasil-L-val column, 25 m×0.25 mm I.D. and 0.12 μm film thickness (Chrompack, Bergen op Zoom, Netherlands). The chromatography was isothermally performed at 195°C. Helium was used as the carrier gas at a flow-rate of 3 ml/min. The remaining chromatographic conditions were as follows: temperature injector site, 200°C; flow detector gases, air at 300 ml/min, hydrogen at 30 ml/min, make-up gas, helium at 30 ml/min; split injection 1:50. The components were detected with a nitrogen–phosphorous-selective flame ionisation detector (NPD) at a temperature of 250°C. The detector was connected to a Model DP 700 integrator (Carlo Erba Instruments). Generally a split ratio of 1:50 was used. However, for the determination of the limit of detection split ratios of 1:25 for urine samples and of 1:10 for plasma samples were used.

2.3. Methods

Aqueous solutions of both enantiomers of IF, 2- or 3-dechloroethylIF, obtained after chiral HPLC of the racemic compounds were prepared, immediately analysed by chiral GC and stored at 4°C and −20°C in 50-μl aliquots. The same procedure was followed for determining the potential racemisation of the enantiomers in plasma and urine.

Samples for the calibration graphs were composed of 50-μl portions of plasma or urine to which 10–50 μl of an aqueous solution of the racemic compounds were added. When the total volume did not add up to 100 μl, the sample was supplemented with water. The *R/S* ratios of the various compounds contained in the stock solution were established by HPLC. The samples were then processed as described earlier [24,25].

In order to determine the recoveries of the various compounds, aliquots of an aqueous solution with a known concentration of the racemic compound were added to plasma or urine samples. The *R/S* ratios in the aqueous solutions were established by chiral HPLC. Subsequently, the spiked plasma and urine samples were processed without adding the internal standard. After evaporation of the ethyl acetate extract, the residue was dissolved in 50 μl ethyl acetate containing TF as the internal standard. The recovery was then assessed by comparing the AUC ratios in the chromatogram with a calibration graph from the racemic mixtures in ethyl acetate.

2.4. Patient samples

The method was used to determine the concentration and enantiomeric ratio of IF, 2- and 3-dechloroethylIF in a 44-year old male patient treated for a rectal carcinoma with a 10-day continuous infusion of IF at a dose of 2500 mg/day. Blood samples were collected into heparinised tubes. The plasma was separated by centrifugation (10 min at 3500 rpm). The plasma samples were stored at −20°C until analysis. Blood samples were taken before starting the infusion and after 8, 12, 24 h and every consecutive day up to 11 days after the start of the treatment. Urine samples were collected as 24-h urine. Until the 24-h portions were completed they were kept at 4°C. After the collection the total volume was measured and 10-ml aliquots were taken and stored at −20°C until analysis.

3. Results and discussion

3.1. Enantiomeric separation and identification

Attempts to obtain enantioselectivity after derivatisation with anhydrous chloral according to the method of Reid et al. [26] for the IF analogue cyclophosphamide (CP) were unsuccessful for IF. Furthermore, separation was also not accomplished

when cyclodextrins were added to the mobile phase of a HPLC reversed-phase system.

However, enantioselective separation could be achieved of all enantiomers of the parent drug IF and its dechloroethylated metabolites using a Chiralcel HPLC column. This column consists of cellulose material derivatised with dimethylphenylcarbamate. The separation is based on a multi-mode mechanism of hydrogen bonding, $\pi-\pi$ interactions, dipole stacking and/or inclusion complexes. The elution order was in concordance with earlier reports from Masurel et al. [21]. However, these investigators did not report the capacity factors (k') of the dechloroethylated metabolites. Because the pure enantiomers were not available, the enantiomers of 2- and 3-dechloroethylIF were isolated by repeatedly injecting a concentrated solution of the racemic compound into the HPLC system. The separated isomers were then collected and the organic solvent was evaporated. An aqueous solution of the separate enantiomers was analysed with a micro-polarimeter and the direction of the rotation was established. The racemic 3-dechloroethylIF did not show complete baseline separation of the enantiomers, but it was still possible to collect the enantiomers containing less than 1% impurity of the opposite enantiomer as verified by HPLC analysis of the isolated products. The k'-values for the enantiomers are summarised in Table 1. The laevorotary isomers of IF and 2-dechloroethylIF possess the *S* configuration, while, according to Cahn–Ingold–Prelog, the (−)-isomer of 3-dechloroethylIF is designated the *R* configuration [27].

The Chirasil-L-val column, used in the GC analysis in this study, consists of a silicon-based polymer with a chiral selector (L-valine) incorporated into the polymer, similar to the method of Young et al. [18]. The elution order of the various enantiomers on the GC column was established by injection of the pure enantiomers obtained after HPLC separation. Although TF possesses a chiral center, the enantiomers were not separated on this column, making TF a suitable internal standard for the GC measurements.

Table 1
Chromatographic parameters for the enantiomers of ifosfamide and metabolites

Compound	GC Chirasil-L-val			HPLC Chiracel OD		
	k'_S	k'_R	α	k'_S	k'_R	α
IF	15.50	16.12	1.04	2.53	1.70	1.49
2-DechloroethylIF	8.44	8.72	1.03	3.33	2.17	1.53
3-DechloroethylIF	15.11	16.54	1.09	2.75	2.50	1.10
Trofosfamide	17.03	17.03	1.00	–	–	–

k'_S: capacity factor for the (*S*) enantiomer; k'_R: capacity factor for the (*R*) enantiomer; α: stereoselectivity.

The data from Table 1 show that in HPLC the *R*-isomers elute first, while in GC the *S*-isomers are the first eluted compounds.

The presented method shortens the time of analysis by one half compared to some other reported GC methods [17,27], while it is comparable to the method of Granville et al. [19].

3.2. Validation

The concentrations of the enantiomers in aqueous and biological samples, prepared as described, did not change after an incubation period of eight weeks. Consequently, in vitro racemisation does not have to be taken into consideration.

Boos et al. [28] established the total IF, 2-and 3-dechloroethylIF concentration by achiral GC, whereas the enantiomeric composition of the samples was determined by chiral GC. This approach requires a double analysis of each sample. The same applies for the HPLC method developed by Corlett and Chrystyn [23] for enantiomeric IF analysis, where an achiral and a chiral system are coupled. The sensitivity of this method (detection limit approx. 2.5 μg/ml) is totally unsatisfactory for bioanalytical and pharmacokinetical research. Moreover, the main IF metabolites were not included in the study.

With the presented method the determination of the concentration of IF and its dechloroethylated metabolites and the enantiomeric composition can be accomplished in one run. The chiral method was validated and the validation parameters are summarised in Tables 2–5. Considering the fact that most physical properties, like solubility, volatility etc., of enantiomers are the same, it is obvious that the variation in the recovery and accuracy for the *R*- and *S*-forms of all compounds fell within the variation of the method. The calibration graphs of the two enantiomers of each compound are well in agreement. Furthermore, the extraction recoveries of the *R*- and *S*-forms are similar. The protein binding of IF

Table 2
Validation parameters of the analysis of the *R*-enantiomers of ifosfamide (IF), 2- and 3-dechloroethylifosfamide (2 and 3, respectively) in plasma ($n=10$)

Concentration (μg/ml)	Recovery (%)			Accuracy (%)			Precision (%)					
							Intra-day			Inter-day		
	IF	2	3	IF	2	3	IF	2	3	IF	2	3
1.0	88.0 (±2.3)	44.9 (±2.0)	72.0 (±2.3)	99.5	98.9	97.2	4.8	3.9	4.2	5.2	4.6	4.9
10.0	90.0 (±2.2)	45.2 (±1.8)	69.7 (±2.2)	99.7	99.2	97.4	4.9	4.0	4.3	5.3	4.5	4.9
100.0	90.3 (±1.7)	45.0 (±1.9)	70.4 (±2.1)	100.2	99.0	98.9	4.9	3.9	4.1	5.1	4.5	4.8

Compound	Calibration graph	Correlation coefficient	Limit of detection (ng/ml)
(+)-(*R*)-Ifosfamide	$y=(-0.052\pm0.025)+(0.197\pm0.002)x$	0.9995 (0–25 μg/ml)	20
(+)-(*R*)-2-Dechloroethylifosfamide	$y=(-0.011\pm0.006)+(0.061\pm0.001)x$	0.9994 (0–10 μg/ml)	100
(−)-(*R*)-3-Dechloroethylifosfamide	$y=(-0.047\pm0.010)+(0.039\pm0.001)x$	0.9995 (0–20 μg/ml)	50

Table 3
Validation parameters of the analysis of the *S*-enantiomers of ifosfamide (IF), 2- and 3-dechloroethylifosfamide (2 and 3, respectively) in plasma ($n=10$)

Concentration (μg/ml)	Recovery (%)			Accuracy (%)			Precision (%)					
							Intra-day			Inter-day		
	IF	2	3	IF	2	3	IF	2	3	IF	2	3
1.0	89.0 (±2.3)	44.3 (±2.1)	72.5 (±2.3)	99.2	97.4	97.4	5.0	3.9	4.2	5.2	4.8	4.9
10.0	90.2 (±2.1)	45.5 (±1.9)	69.5 (±2.0)	98.7	99.8	97.1	4.9	4.2	4.0	5.2	4.4	4.9
100.0	90.3 (±1.9)	44.8 (±1.9)	70.2 (±2.3)	99.8	99.1	98.9	4.9	4.3	4.1	5.3	4.5	4.8

Compound	Calibration graph	Correlation coefficient	Limit of detection (ng/ml)
(−)-(*S*)-Ifosfamide	$y=(-0.049\pm0.026)+(0.197\pm0.002)x$	0.9997 (0–25 μg/ml)	20
(−)-(*S*)-2-Dechloroethylifosfamide	$y=(-0.0096\pm0.005)+(0.061\pm0.001)x$	0.9994 (0–10 μg/ml)	100
(+)-(*S*)-3-Dechloroethylifosfamide	$y=(-0.065\pm0.014)+(0.039\pm0.001)x$	0.9994 (0–20 μg/ml)	50

Table 4
Validation parameters of the analysis of the *R*-enantiomers of ifosfamide (IF), 2- and 3-dechloroethylifosfamide (2 and 3, respectively) in urine ($n=10$)

Concentration (μg/ml)	Recovery (%)			Accuracy (%)			Precision (%)					
							Intra-day			Inter-day		
	IF	2	3	IF	2	3	IF	2	3	IF	2	3
1.0	86.0 (±2.4)	45.4 (±2.5)	70.2 (±2.5)	100.1	98.5	97.1	4.8	4.2	4.3	5.2	5.0	4.9
10.0	90.0 (±2.0)	46.1 (±2.1)	71.6 (±2.2)	99.9	98.3	100.2	4.9	4.0	4.1	5.4	4.9	5.0
100.0	90.1 (±1.6)	45.1 (±2.0)	69.9 (±1.9)	101.0	99.1	99.8	5.0	4.6	4.7	5.0	4.9	4.9

Compound	Calibration graph	Correlation coefficient	Limit of detection (ng/ml)
(+)-(*R*)-Ifosfamide	$y=(-0.033\pm0.09)+(0.020\pm0.0006)x$	0.9990 (0–250 μg/ml)	50
(+)-(*R*)-2-Dechloroethylifosfamide	$y=(-0.039\pm0.016)+(0.0078\pm0.0003)x$	0.9986 (0–50 μg/ml)	150
(−)-(*R*)-3-Dechloroethylifosfamide	$y=(-0.073\pm0.044)+(0.0044\pm0.0004)x$	0.9980 (0–150 μg/ml)	75

Table 5
Validation parameters of the analysis of the *S*-enantiomers of ifosfamide (IF), 2- and 3-dechloroethylifosfamide (2 and 3, respectively) in urine ($n=10$)

Concentration (μg/ml)	Recovery (%)			Accuracy (%)			Precision (%)					
							Intra-day			Inter-day		
	IF	2	3	IF	2	3	IF	2	3	IF	2	3
1.0	86.1 (±2.3)	45.7 (±2.4)	70.0 (±2.5)	98.7	98.9	97.0	4.8	4.2	4.2	5.3	5.0	4.9
10.0	90.1 (±2.2)	45.9 (±2.2)	71.9 (±2.3)	101.0	97.6	99.6	5.0	4.3	4.3	5.3	5.1	5.0
100.0	89.8 (±1.8)	45.1 (±1.9)	70.0 (±2.1)	100.1	99.1	100.3	5.0	4.5	4.8	5.3	5.0	5.0

Compound	Calibration graph	Correlation coefficient	Limit of detection (ng/ml)
(−)-(*S*)-Ifosfamide	$y=(-0.036\pm0.09)+(0.020\pm0.0006)x$	0.9990 (0–250 μg/ml)	50
(−)-(*S*)-2-Dechloroethylifosfamide	$y=(-0.026\pm0.015)+(0.0075\pm0.0003)x$	0.9985 (0–50 μg/ml)	150
(+)-(*S*)-3-Dechloroethylifosfamide	$y=(-0.084\pm0.037)+(0.0049\pm0.0005)x$	0.9980 (0–150 μg/ml)	75

is negligible, that of the dechloroethylated compounds, however, is unknown. Due to the higher polarity of 2- and 3-dechloroethylIF compared to IF, the extraction recoveries of the metabolites were less than that of the parent drug. The more polar 2-dechloroethylIF was less recovered from urine than 3-dechloroethylIF.

The accuracy was established by ten analyses of a urine or plasma sample of three known concentrations of the racemic compounds. The *R*/*S* ratios of

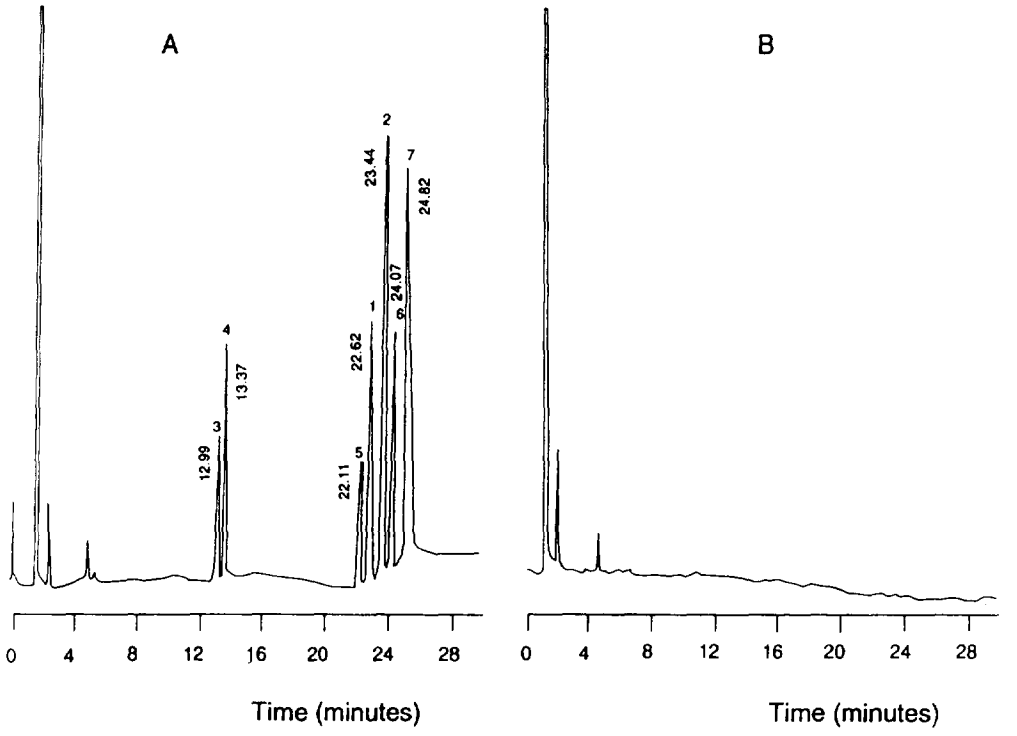

Fig. 2. Gas chromatogram of (A) a patient urine sample containing 20.6 μg/ml IF, 20.2 μg/ml 2-dechloroethylIF and 46.1 μg/ml 3-dechloroethylIF and (B) a drug-free urine sample. Peaks: 1=(−)-(*S*)-IF, 2=(+)-(*R*)-IF, 3=(−)-(*S*)-2-dechloroethylIF, 4=(+)-(*R*)-2-dechloroethylIF, 5=(+)-(*S*)-3-dechloroethylIF, 6=(−)-(*R*)-3-dechloroethylIF and 7=trofosfamide.

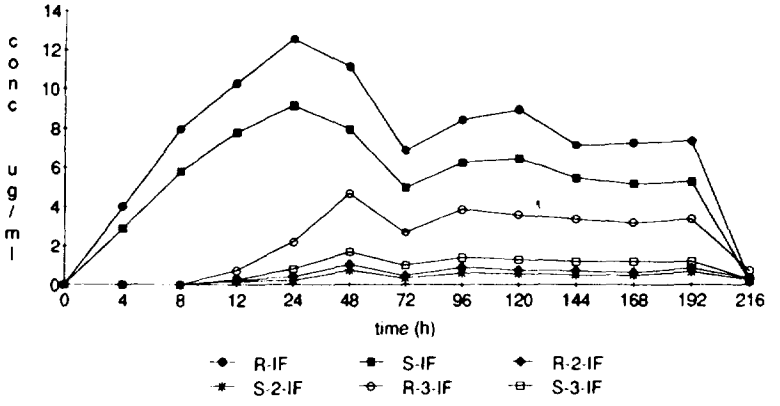

Fig. 3. Plasma concentration–time curve of IF, 2- and 3-dechloroethylIF of patient with a 10-day continuous infusion of 2500 mg IF per day.

the compounds in the aqueous standard solutions and, thereby, the concentrations of the *R*- and *S*-isomers, were determined by HPLC. The concentration of the sample calculated by means of a calibration graph was then compared with its exact concentration.

3.3. Patient samples

A gas chromatogram of a patient urine sample is shown in Fig. 2. This chromatogram reveals a urinary enrichment of *R*-IF, *R*-2-dechloroethylIF and *R*-3-dechloroethylIF. A plasma concentration–time curve for this patient is shown in Fig. 3. A similar enantiomeric preference can be observed. These results may indicate a stereospecific metabolism of IF and necessitate further investigations by means of the described method.

4. Conclusions

The presented chiral GC method is suitable to determine the total concentration and the enantiomeric composition of IF and its dechloroethylated metabolites in one single run. The results of the analysis of a patient's urine samples indicate stereospecific metabolism of IF. The presented method is being used to study the metabolism of IF in more detail.

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