Journal of Chromatography, 583 (1992) 175–182
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6579

Gas chromatographic determination of 2- and 3-dechloroethylifosfamide in plasma and urine

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(First received May 21st, 1992; revised manuscript received September 9th, 1992)

ABSTRACT

The metabolic oxidation of one of the chloroethyl groups of the antitumour drug ifosfamide leads to the formation of the inactive metabolites 2- and 3-dechloroethylifosfamide together with the neurotoxic metabolite chloroacetaldehyde. A very sensitive capillary gas chromatographic method, requiring only 50 μl of plasma or urine, has been developed to measure the amounts of the drug and the two inactive metabolites in a single run. Calibration curves were linear ($r > 0.999$) in the concentration ranges from 50 ng/ml to 100 μg/ml in plasma and from 100 ng/ml to 1 mg/ml in urine.

INTRODUCTION

The antitumour drug ifosfamide [IF, 3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine 2-oxide] belongs to the oxazaphosphorines. Together with its structural analogue cyclophosphamide (CP), IF is widely used in the chemotherapy of various forms of cancer [1]. Dosages of IF of *ca.* 7.5–15 g over a period of 1–3 days are typically used for the treat-

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0378-4347/92/$05.00

ment of various froms of cancer, *e.g.* soft tissue sarcomas.

The oxazaphosphorine drugs are inactive as such and need to be activated in the liver. Prior to the formation of the alkylating agent (iso)phosphoramide mustard, they show a complex pattern of metabolism. Side-chain oxidation of the chloroethyl groups at the exocyclic and cyclic nitrogen of IF is a competitive mechanism leading to the formation of 2- and 3-dechloroethylifosfamide (2- and 3-DClIF), respectively, both inactive metabolites. Dechloroethylation of IF also leads to the formation of equimolar amounts of chloroacetaldehyde, which is held responsible for the neurotoxic side-effects of IF therapy [2–6]. A high-performance liquid chromatographic (HPLC) method for the analysis of chloroacetaldehyde has been reported [7]. This method, however, needs special sample pretreatment because of the instability of chloroacetaldehyde.

Knowledge of the proportion of IF that is immediately deactivated to 2- and 3-DClIF, is essential to gain insight into the mechanisms underlying the undesired inactivation of the drug and generation of the neurotoxic metabolite.

Various authors have used thin-layer chromatography (TLC) [8–10], gas chromatography (GC) [11–15] or HPLC [16] to determine 2- and 3-DClIF in urine and/or plasma. ^{31}P nuclear magnetic resonance (NMR) spectroscopy has also been used to quantitate IF, and 2- and 3-DClIF in urine samples [17–19]. The detection limits of HPLC are high because of the poor UV absorbance qualities of IF and 2- and 3-DClIF (absorbance maximum at 190 nm, $\varepsilon = 1000$). The reported GC methods [11–15] show low detection limits for underivatized IF.

This paper reports the GC analysis of 2- and 3-DClIF in plasma and urine. The method has been validated and has proved to be useful for the analysis of patients' samples. The major advantages of the procedure are that it is simple, fast and sensitive, and only 50-μl samples are required, which is especially useful in pediatric oncology. This report is a sequel to an earlier study focused on the analysis of IF [13].

EXPERIMENTAL

Chemicals

2- and 3-DClIF and trofosfamide (TF) were kindly donated by Asta Medica (Frankfurt, Germany). IF was purchased from Dagra Pharma (Diemen, Netherlands). The substances were used as received. The identity of the substances was checked with infrared spectroscopy. The purity was established with HPLC and GC and appeared to be >99%. Analytical-reagent grade sodium hydroxide and ethyl acetate were purchased from Merck (Darmstadt, Germany) and were of the highest purity available. All other chemicals were of analytical grade and used as received. Throughout the study distilled water was used.

Instrumentation

The instrumentation and the applied temperature programme are as described previously [13]. A gas chromatograph (HRGC 5300, Carlo Erba Instruments, Milan, Italy) equipped with a split–splitless injector and nitrogen–phosphorus-selective flame ionization detection (NP-FID) was used. Separation was achieved on a capillary column (CP-Sil 8 CB, 25 m × 0.32 mm I.D., film thickness 0.12 μm, Chrompack, Bergen op Zoom, Netherlands). The starting temperature of the column was 120°C and was raised to 200°C at 40°C/min, where it was held at 200°C for 8 min.

The chromatograph was operated under the following conditions: temperature at the injection site, 175°C; temperature of the detector, 275°C; carrier gas, helium, at a flow-rate of 3 ml/min; flow detector gases, air at 300 ml/min, hydrogen at 30 ml/min, make-up gas, helium, at 30 ml/min; split injection, where the split depends on the concentration in the sample. The detector was connected to a Model DP 700 integrator (Carlo Erba Instruments).

For GC measurements with mass spectrometric (MS) detection a Finnigan 700 ion-trap detector (Finnigan MAT, San José, CA, USA) was used, operated in the chemical ionization (CI) mode with isobutane as the CI reagent gas. The manifold temperature was maintained at 200°C,

and the temperature of the transfer line was held at 260°C. The detector was operated in the automatic reaction control (ARC) mode.

Plasma clean-up procedure

The internal standard used was TF. Solutions of TF and 2- and 3-DClIF were made up in water at various concentrations. Standard solutions for the preparation of plasma samples for the calibration line contained both 2- and 3-DClIF, and were stored at 4°C. Drug-free plasma samples were obtained from healthy volunteers.

The clean-up procedure of the plasma samples was as described by Kaijser *et al.* [13]. For the concentration range 10–1000 ng/ml plasma a split ratio of 1:10 was used; for the concentration range 1–100 μg/ml plasma the split had to be 1:50.

Samples for the calibration curves were composed of 50 μl of drug-free plasma, to which different amounts of an aqueous solution of 2- and 3-DClIF were added. This was supplemented with distilled water to a total volume of 100 μl. Calibration samples were treated identically with the corresponding patients' samples, yielding a final solution of 135 μl, which was then processed in the same manner.

Urine clean-up procedure

Urine samples were treated in the same way as plasma samples. Drug-free urine samples were obtained from healthy volunteers.

In the whole concentration range (10 ng to 1 μg 2- and 3-DClIF per ml) 50 μl of urine were used and the dry residue, obtained after extraction and evaporation of the organic extract, was dissolved in 20 μl of ethyl acetate.

For the concentration range 100 ng to 1 μg 2- and 3-DClIF per ml a split ratio of 1:25 was used; for the range 1–1000 μg the split ratio was 1:50.

Chemical stability

Standard solutions of TF and 2- and 3-DClIF in water at a concentration of 100 ng/ml, and in ethyl acetate at a concentration of 10 μg/ml, were kept in the refrigerator (4°C) for 8 weeks, after which time they were analysed.

Before analysis, solutions of 2- and 3-DClIF in plasma at concentrations of 100 ng/ml and 10 μg/ml, and in urine at concentrations of 1 and 10 μg/ml, were kept at room temperature for 5 h and stored in the refrigerator (4°C) and in the freezer (−20°C); the samples were analysed after 4 and 8 weeks. Plasma and urine samples of the same concentrations were also kept at room temperature for 2 h after preparation of the sample. Next, the samples were stored in the freezer (−20°C) for 4 weeks. Before analysis the samples were kept at room temperature for 2 h.

The stability of plasma samples containing 100 ng/ml and 10 μg/ml 2- and 3-DClIF was also investigated after 30 min in a thermostatically controlled water-bath at a temperature of 60°C; this procedure has been advocated for the inactivation of viruses, *e.g.* human immunodeficiency virus (HIV) [20].

Gas chromatography–mass spectrometry

GC–MS studies were performed with patients' urine samples containing *ca.* 175 μg of 2-DClIF per ml and 500 μg of 3-DIClIF per ml. The samples were processed as described above except for the volume of ethyl acetate in which the dry residue was dissolved. For this purpose the residue was dissolved in 10 μl of ethyl acetate, and 1 μl of the final solution (*ca.* 0.8 μg of 2-DClIF and 2.5 μg of 3-DClIF per ml) was injected into the gas chromatograph at a split ratio of 1:10. The same temperature programme as described above was applied.

Potential chromatographic interferences of related compounds were checked by injecting a solution of keto-ifosfamide, carboxy-ifosfamide and 2,3-didechloroethylifosfamide into the chromatograph.

Patients' samples

The method described above was used to determine the plasma concentration of 2- and 3-DClIF together with the concentration of IF, in a chemotherapy naive 60-year-old male patient with advanced colon cancer. He received a continuous IF infusion with a dose of 2 g/day during 10 consecutive days with concomitant adminis-

tration of sodium 2-mercaptoethanesulphonate. Cycles were repeated at 4 weeks. The procedure for the analysis of IF is the same as the procedure for 2- and 3-DClIF, described previously [13].

Samples were taken before starting the infusion and 5, 10, 20 and 40 min, and 1, 2, 3, 4, 8 and 12 h after the start of the infusion, and after 1, 2, 3, 4, 5, 7, 9, 10 and 11 days.

Urine samples were collected for 11 days at 24-h intervals, and 8-ml aliquots were frozen at −20°C prior to analysis.

RESULTS AND DISCUSSION

Extraction

For the extraction of IF and CP from biological fluids several methods have been described [13–16, 21–24]. Extraction recoveries of 2- and 3-DClIF are often not reported. The liquid–liquid extraction (LLE) that Goren [16] modified after Margison *et al.* [22], and the solid-phase extraction (SPE) method of Momerency *et al.* [24], appeared to have the highest extraction recovery for 2- and 3-DClIF (75% and 80%, respectively), whereas all LLE and SPE methods have comparable extraction recoveries for IF and CP (>95%). The SPE method developed by Hardy *et al.* [21] showed a recovery of 91% for IF, but only 40% recovery for both 2- and 3-DClIF. The LLE method of Goren [16], using chloroform as the extraction solvent, is a rather elaborate method including various filtration steps, and was validated only for 3-ml urine samples. LLE of 2- and 3-DClIF from alkalinized urine and plasma samples with ethyl acetate was preferred because this method was already completely validated, and proved satisfactory, for the extraction of IF. This method is used for routine analysis of IF in our laboratory and allows us to determine IF and 2- and 3-DClIF simultaneously in a single run [13].

Washing the ethyl acetate extract with hexane after evaporation and reconstitution in methanol has been advocated to prolong the lifetime of the GC column [25]. The column used in our laboratory, however, does not show a degradation of the separation efficiency after a one-year use without the use of a retention gap.

Chromatography

Fig. 1 shows a typical chromatogram for the assay of IF, 2- and 3-DClIF in a patient's plasma sample. The retention times for IF and 2- and 3-DClIF are 4.37, 2.87 and 3.15 min, respectively.

The two metabolites of IF that are present in patients' plasma and urine samples showed equal retention times to the reference compounds 2- and 3-DClIF. Moreover, GC–MS measurements showed that the ^{35}Cl isotope-containing metabolites have a molecular mass of 198 (MH^+ 199, Fig. 2). The spectra belong to a fragment containing a single Cl atom indicated by a ratio of three of the peaks at 199 (^{35}Cl isotope) and at 201 (^{37}Cl isotope), reflecting the natural occurrence of the Cl isotopes. Further fragmentation appeared to an extent of less than 5%. The fragment shows a molecular mass of 163 (MH^+ 164), indicating the loss of the last chloroethyl group resulting in 2,3-didechloroethylifosfamide. These results, together with the fact that carboxy-ifosfamide, keto-ifosfamide and 2,3-didechloroethylifosfamide do not interfere with the 2- and 3-dechloroethylifosfamide peaks, prove that the metabolites indeed are 2- and 3-DClIF and that both compounds were chromatographed unchanged.

Validation

TF is used as the internal standard because it is thermally more stable than CP [13]. Calibration graphs were made in decades. The overall calibration graphs in plasma and urine are shown in Table I with correlation coefficients exceeding 0.999.

Percentage recoveries of known amounts of 2- and 3-DClIF in plasma and urine were calculated for each calibration curve, at a concentration in the middle of the graph, by measuring the absolute amount of 2- and 3-DClIF recovered, using a calibration curve of 2- and 3-DClIF in ethyl acetate. The recoveries from plasma in the various calibration ranges were 44.3(±0.9)% for 2-DClIF and 68.9(±0.5)% for 3-DClIF. In urine the recoveries were 43.6(±0.9)% for 2-DClIF and 69.6(±0.4)% for 3-DClIF.

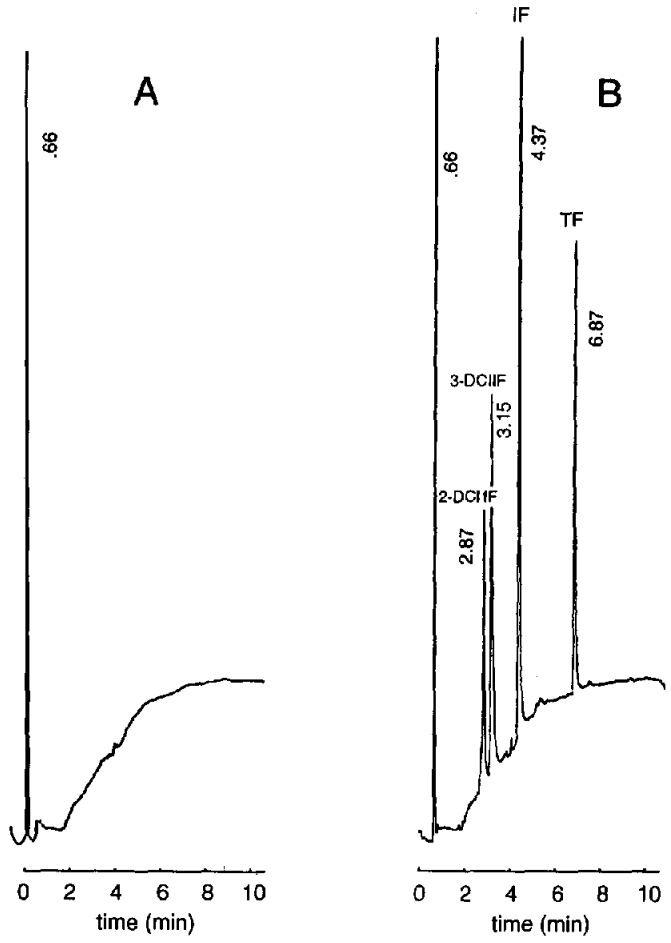

Fig. 1. Chromatograms of (B) a patient's plasma sample containing 34 μg of IF per ml, 3.5 μg of 2-DClIF per ml and 10 μg of 3-DClIF per ml (split 1:50), and (A) a drug-free patient's sample.

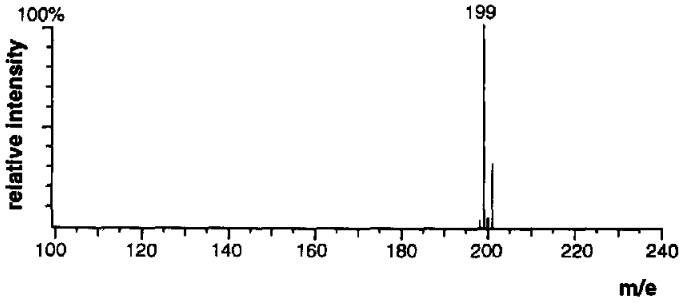

Fig. 2. Mass spectrum of chromatographed 2-DClIF in urine (the mass spectrum of 3-DClIF is identical).

TABLE I

OVERALL CALIBRATION GRAPHS FOR 2- AND 3-DCIIF IN PLASMA AND URINE

y represents the ratio of peak areas of the analyte and the internal standard TF, and x is the concentration of the analyte.

2-DCIIF in plasma (50 ng–100 μg/ml)	$y = -0.089(\pm 0.06) + 0.039(\pm 0.003)x$; $r = 0.9992$
3-DCIIF in plasma (50 ng–100 μg/ml)	$y = -0.088(\pm 0.04) + 0.032(\pm 0.001)x$; $r = 0.9993$
2-DCIIF in urine (100 ng–1 mg/ml)	$y = -0.068(\pm 0.09) + 0.037(\pm 0.003)x$; $r = 0.9991$
3-DCIIF in urine (100 ng–1 mg/ml)	$y = -0.095(\pm 0.03) + 0.036(\pm 0.001)x$; $r = 0.9996$

The accuracy and intra- and inter-assay precision were determined in the middle of the various decades of the whole concentration range for plasma and urine, and are summarized in Tables II and III, respectively.

To determine the intra-assay precision, five analysed samples with a concentration in the middle of the calibration graph and measured on the same day were compared. To determine the inter-assay precision, a sample with a concentration in the middle of each calibration curve was quantified on five consecutive days.

TABLE II

ACCURACY, INTRA- AND INTER-ASSAY PRECISION OF THE DETERMINATION OF 2- AND 3-DCIIF IN PLASMA (n = 5)

Concentration	Accuracy (%)		Intra-assay precision (%)		Inter-assay precision (%)	
	2-DCIIF	3-DCIIF	2-DCIIF	3-DCIIF	2-DCIIF	3-DCIIF
50 ng/ml	90.1	92.3	2.6	2.3	3.5	2.5
500 ng/ml	97.0	98.1	2.2	1.9	2.7	2.1
5 μg/ml	100.0	99.9	1.9	1.7	2.5	1.9
50 μg/ml	99.7	99.9	2.0	1.9	2.1	2.2

TABLE III

ACCURACY, INTRA- AND INTER-ASSAY PRECISION OF THE DETERMINATION OF 2- AND 3-DCIIF IN URINE (n = 5)

Concentration	Accuracy (%)		Intra-assay precision (%)		Inter-assay precision (%)	
	2-DCIIF	3-DCIIF	2-DCIIF	3-DCIIF	2-DCIIF	3-DCIIF
50 ng/ml	97.6	95.5	2.6	2.1	2.7	2.3
500 ng/ml	100.5	99.7	1.2	0.9	1.9	1.2
5 μg/ml	100.1	100.2	0.9	0.9	1.0	1.1
50 μg/ml	99.8	99.1	1.0	1.1	1.2	1.5

The limit of detection (signal-to-noise ratio of 3:1), when using a split ratio of 1:10 and an injection volume of 1 μl, appears to be 10 ng of 3-DClIF per ml plasma, 30 ng of 2-DClIF per ml plasma, 25 ng of 3-DClIF per ml urine and 70 ng of 2-DClIF per ml urine.

It is concluded that the presented procedure is a suitable method for the determination of IF and 2- and 3-DClIF levels in biological fluids. Because the analytical method covers a large concentration range, it was not possible to use a single 2- and 3-DClIF and TF stock solution without exceeding 75 μl for addition. Therefore, TF and 2- and 3-DClIF solutions of different concentrations were used for the preparation of the calibration samples.

Chemical stability

The concentrations of 2- and 3-DClIF and TF in water and ethyl acetate and the concentrations of 2- and 3-DClIF in plasma and urine used to test the chemical stability of the analytes were chosen in accordance with the concentrations in patient samples and the solutions used for the analysis. Solutions of 2- and 3-DClIF in plasma and urine, stored in the refrigerator, showed a loss of *ca.* 10% after 4 weeks but were stable when stored at −20°C. All other solutions appeared to be stable under the reported circumstances. Stock solutions of 2- and 3-DClIF in water and ethyl acetate can, therefore, be stored at 4°C, whereas urine and plasma samples containing 2- and 3-DClIF should be kept at a temperature of −20°C, at which temperature the metabolites are stable for at least 8 weeks.

Solutions containing both 2- and 3-DClIF in water also did not show a change in concentration during storage. Consequently, stock solutions for the determination of calibration graphs can be prepared containing both compounds.

Patients' samples

The chromatogram of a patient's plasma sample (Fig. 1) shows that the described method allows simultaneous determination of IF and 2- and 3-DClIF.

The results of the analysis of IF and 2- and 3-DClIF in a patient's sample is shown in Fig. 3. Both metabolites appeared in the plasma samples 12 h after the start of the infusion. At the plateau in the plasma concentration–time curve the sum of the concentrations of 2- and 3-DClIF is about one-third of the IF concentration. The plasma concentration of 3-DClIF appeared to be three times higher than the concentration of 2-DClIF. In most patients the same ratio of 3-DClIF to

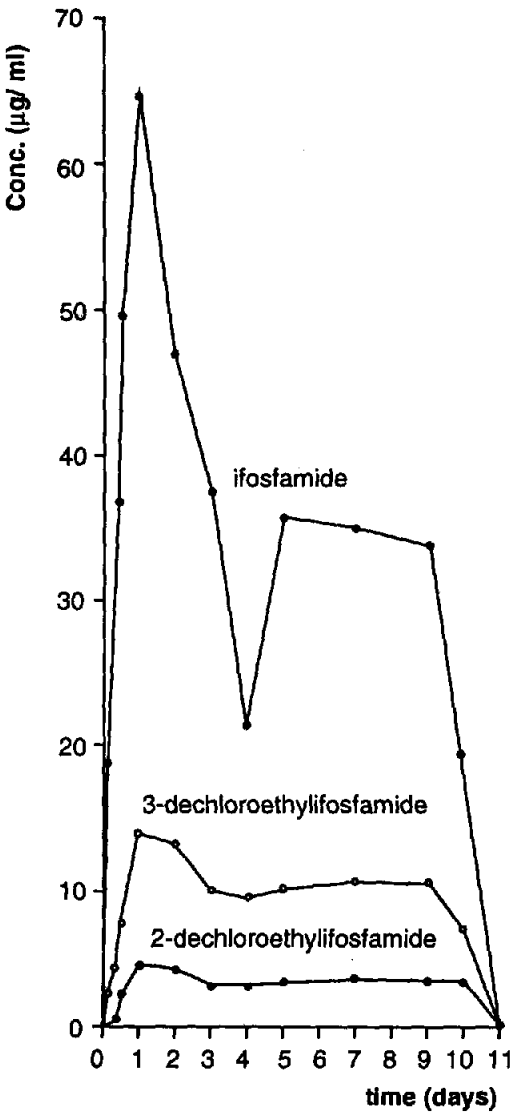

Fig. 3. Plasma concentrations of IF and 2- and 3-DClIF in a patient receiving a continuous daily infusion of 2 g of IF for 10 days.

2-DClIF was found for the excretion in urine. However, a large inter-patient variation in side-chain oxidation exists: in some patients receiving a 10-day continuous infusion of 2 g IF/day the ratio of 3-DClIF/2-DClIF mounts up to 12. The ratio of the cumulative amount of the two metabolites to the amount of IF, excreted in the urine, varies from 0.2 to 2.7. This is consistent with the findings of Goren [16], who reported a ratio of 0.7–2.8, although only patients were studied who were given a single IF dose of 1.6 g/m^2 in 15 min. The sum of 2- and 3-DClIF excreted in the urine is found to be no higher than 19% of the total IF dose, whereas values of 11–37% [14], 15–30% [16], 19–33% [18] and 48% [11] have been reported by other authors. The reason for the relatively low percentage found in this study may be related to different administration schedules.

The total amount of unchanged IF excreted in the urine appeared 3–13%, indicating a minimum loss of 22–32% of IF potency.

CONCLUSION

The presented method is a simple one for the simultaneous analysis of IF and 2- and 3-DClIF without prior derivatization. The method is faster and more suitable for the observation of changes in IF metabolism or saturation of the deactivation of IF into 2- and 3-DClIF on different IF treatments than a separate HPLC analysis of the very unstable chloroacetaldehyde and GC IF analysis.

ACKNOWLEDGEMENT

The authors thank Mr. R. D. van Ooijen for his technical assistance.

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