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Determination of 4-hydroxyifosfamide in biological matrices by high-performance liquid chromatography

G.P. Kaijser [a], P.G.J.H. Ter Riet [a], J. de Kraker [b], A. Bult [a], J.H. Beijnen [c], W.J.M. Underberg [a,*]

[a] *Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[b] *Academic Medical Centre, University Hospital of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands*
[c] *Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

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Abstract

A high-performance liquid chromatographic method has been developed for the determination of 4-hydroxyifosfamide, a metabolite of ifosfamide, in plasma of cancer patients. The analyte is derivatized to 7-hydroxyquinoline, which can be detached fluorimetrically. The calibration graph is linear in the concentration range 0.05–25 μM, the limit of detection being 40 nM. Any inference from acrolein, another metabolite of ifosfamide, was ruled out. 4-Hydroxyifosfamide is very unstable in plasma and a stabilization procedure by adding citric acid has been developed. Thus treated, the samples were stable for 4 days. Analysis of a patient's plasma samples revealed that the 4-hydroxifosfamide concentration did not exceed 10 μM. © 1997 Elsevier Science B.V.

Keywords: Biological matrices; High-performance liquid chromatography; 4-Hydroxyifosfamide

1. Introduction

4-Hydroxyifosfamide (4-hydroxyIF) is a metabolite of the anti-cancer drug ifosfamide (IF). The route of metabolism of IF, leading to ifosforamide mustard (IFM), the active compound, starts with the hydroxylation of IF to 4-hydroxyIF (Fig. 1) [1]. 4-HydroxyIF is presumed to be in equilibrium with its tautomer aldoifosfamide. However, the latter has never been conclusively demonstrated in vivo [2]. Enzymatic β-elimination leads to IFM and acrolein (Fig. 1). 4-HydroxyIF, which has relatively low alkylating activity, is believed to be taken up by the cells after which IFM is generated, the DNA alkylating species. IFM is too hydrophilic to enter the cells. It is now generally accepted that 4-hydroxylation of oxazaphosphorines is an important step in the bio-activation of these compounds [3–7] and bio-analysis of this compound yields more insight in the essentials of the bio-activation process. Although the important IF metabolites 2- and 3-dechloroethylIF also form 4-hydroxy analogues [8], the amount of these metabolites is only a

* Corresponding author.

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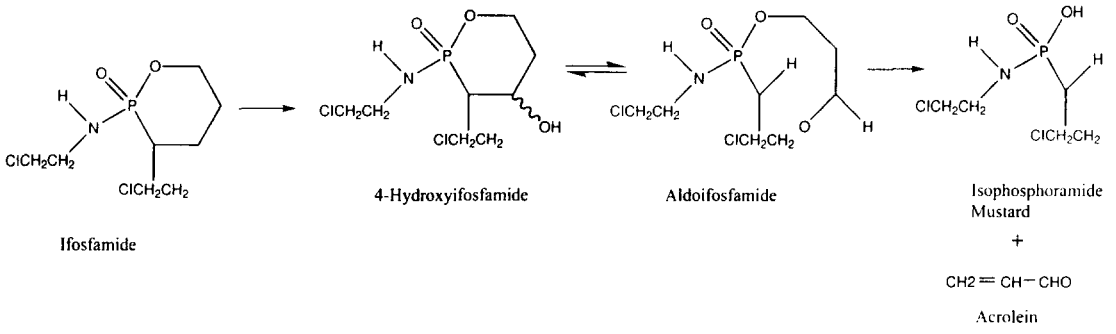


Fig. 1. Activation route in the metabolism of IF.

limited fraction of the totally excreted compound [9]. Considering the low percentage of 4-hydroxylation of IF [10] that can also be expected for the 2- and 3-dechloroethylIF metabolites, the total amount of 4-hydroxy products determinable in biological fluids after IF administration will be a good estimate of the 4-hydroxyIF concentration in these matrices.

The determination of 4-hydroxyIF has been hampered by its instability in biological fluids. 4-Hydroxycyclophosphamide (4-hydroxyCP), the 4-hydroxylated metabolite of the IF analogue cyclophosphamide (CP), has been determined in biological fluids after conversion into a 4-*S*-benzylsuphido- [11] cyanohydrin- [12] or thioglycoside derivative [13]. For both 4-hydroxyCP and 4-hydroxyIF, methods have been described based on the release of acrolein followed by derivatization with 3-aminophenol yielding 7-hydroxyquinoline (HQ), which is amenable to fluorimetric analysis [4,10,11,14–20]. Acrolein, generated in vivo, interferes in these fluorimetric assays. Some investigators have, therefore, extracted samples with dichloromethane with subsequent evaporation of the organic extract in order to remove the volatile acrolein. Acrolein, however, is a very unstable and reactive species that rapidly degrades and binds, almost instantaneously, to plasma components. Therefore, acrolein is extremely difficult to detect in patients' samples, also verified with acrolein-spiked plasma samples. This justifies the use of an assay of 4-hydroxyIF in which the analyte is converted into acrolein in the presence of 3-aminophenol. This reagent traps liberated acrolein by conversion into HQ.

4-HydroxyIF degrades rapidly at room temperature and high pH. Ikeuchi and Amoneo [16] reported that storage of plasma samples at temperatures between 0 and 4°C in a citric acid-phosphate buffer (pH 3.5) is optimal. However, earlier studies reported a catalysing action of phosphate on the degradation of 4-hydroxyoxazaphosphorines where Tris buffers form adducts with the hydroxy derivatives [21–23]. Therefore, the method of choice to prevent 4-hydroxyIF degradation is to acidify the samples with citric acid [16].

Major drawbacks of total fluorescence measurements are that these assays are relatively insensitive with high background signals. Derivization of 4-hydroxyIF in the biological matrix yielding HQ, followed by HPLC analysis, appeared to be more sensitive. We developed an HPLC method for 4-hydroxyIF in plasma after derivatization. The method has been completely validated and the stability of the various compounds was studied as literature data on this subject were limited and confusing.

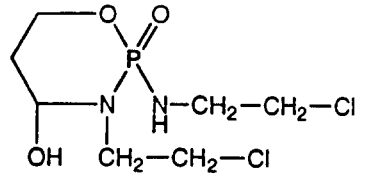
2. Experimental

2.1. Chemicals

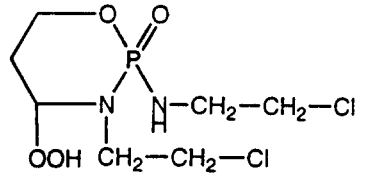
IF, 4-hydroperoxyifosfamide (4-hydroperoxyIF) and mafosfamide (MF) (Fig. 2) were kindly donated by Asta Medica (Frankfurt, Germany).

Acrolein and citric acid were purchased from Merck (Darmstadt, Germany), 3-aminophenol from BHD (Poole, UK), hydroxylamine from Aldrich (Bornem, Belgium), 2,4-dinitrophenylhydrazine (DNPH) from Baker (Deventer, The Netherlands), lidocaine hydrochloride from Sigma (St. Louis, MO, USA) and acetonitrile from Rathburn (Walkerburn, UK). HQ was synthesized by the Skraup reaction with the reagents 3-aminophenol, sulphuric acid, glycerol and sodium 3-nitrobenzenesulphonate [24]. HQ, recrystallized from chlorobenzene, showed a melting point of 238°C. The identity was confirmed by infrared spectroscopy. The purity was established by HPLC with fluorimetric detection and UV detection at 254 nm.

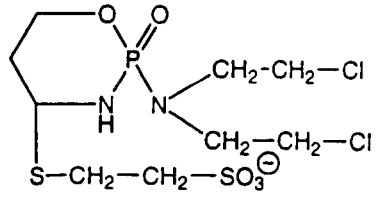
The acrolein-dinitrophenylhydrazine derivate was prepared according to Boor and Ansari [25] by adding 1 ml of a saturated DNPH solution in 2 M sulphuric acid to 250 μl of acrolein. The mixture was stored overnight at 4°C. The next day the yellow crystals were filtered off and recrystallized twice from chloroform.



4-hydroxyifosfamide



4-hydroperoxyifosfamide



mafosfamide

Fig. 2. Structural formulae of 4-hydroxyIF, 4-hydroperoxyIF and mafosfamide.

The buffer solution used to stabilise plasma samples contained 0.2 M citric acid and 0.2% (w/v) lidocaine. HCl (internal standard), adjusted to pH 3.5.

The reaction mixture for the derivatization consisted of 3-aminophenol (0.33%, w/v) and hydroxylamine (antioxidant) (0.4%, w/v) in 2 M hydrochloric acid.

All chemicals were of the highest purity available and were used as received. Throughout the study filtered demineralized water was used (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The HPLC configuration included a Model 510 pump and a U6K injection device, both from Waters (Milford, MA, USA). For the separation a Chromspher C_{18} column (150 × 4.6 mm i.d.) of 5 μm particle size was used (Chrompack, Bergen op Zoom, The Netherlands) protected with a 1 cm reversed-phase (C_{18}) guard column. The mobile phase consisted of a mixture of methanol and an aqueous solution containing 20 mM tetramethylammonium bromide (TMABr) and 5 mM heptanesulphonic acid (10:90, v/v) at pH 3.0 and at a flow rate of 1.0 ml min^{-1}. The mobile phase was filtered (0.2 μm) and degassed before use. Fluorimetric detection was performed with a Separations 821-FP detector (Separations, HI Ambacht, The Netherlands) at an excitation wavelength of 350 nm and an emission wavelength of 510 nm, coupled to a Spectra-Physics integrator (San Jose, CA, USA). This detector could also be used to record on-line the emission and excitation spectra of the eluted compounds.

The UV detector used for the purity control of the synthesized HQ was a Waters Model 440 absorbance detector operating at 254 nm and coupled to a Kipp DB 40 recorder (Kipp and Sons, Delft, The Netherlands).

The HPLC system for the measurement of 4-hydroxyIF after reduction of 4-hydroperoxyIF

consisted of a pump and injection device as described above. A LiChrosorb RP-18 column (125 × 4.0 mm i.d.) of 5 μm particle size was used. The mobile phase was composed of acetonitrile and 10 mM phosphate buffer solution, pH 7.0 (20:80, v/v). For detection a Model 1040A photodiode-array UV detector was used (Hewlett-Packard, Waldbronn, Germany).

The HPLC analysis of derivatized acrolein was performed with the same pump and injection device as described earlier. A LiChroCART RP-18, 5 μm, 125 × 4.0 mm i.d. column (Merck) was used with a mobile phase containing 10 mM sodium phosphate buffer (pH 7.4) and acetonitrile (50:50, v/v). The derivative was detected with a Model 440 absorbance detector (Waters) operating at 365 nm and coupled to a Spectra-Physics integrator.

2.3. Derivatization of 4-hydroxyifosfamide

A 500 μl aliquot of plasma or urine was mixed with 500 μl of the citric acid buffer. To this mixture 1.00 ml reagent solution was added and the mixture was heated at 100°C in a boiling water-bath for 20 min. The solutions were then cooled in melting ice and filtered through a Minisart 0.45 μm filter (Sartorius, Göttingen, Germany) to remove plasma proteins. Subsequently, 10 μl was injected into the chromatographic system.

The calibration graph was constructed starting from 4-hydroperoxyIF or MF. A 100–500 μl aliquot of a solution of 4-hydroperoxyIF in the citric acid buffer solution was added to a 500 μl plasma or urine sample. The total sample volume was adjusted to 1000 μl with the citric acid buffer. These samples were then processed and derivatized as described above. The derivatization was performed for various periods of time (5–30 min) and temperatures (70–100°C) in order to establish the optimum reaction conditions.

2.4. Derivatization method and stability of acrolein

To 1 ml of plasma or urine spiked with acrolein, 1 ml of a saturated DNPH solution in 2 M sulphuric acid was added. The mixture was placed in a water-bath of 37°C for 10 min. Subsequently, the reaction mixture was extracted with 2 ml of chloroform. The layers were separated by centrifugation (5000 rpm for 5 min) and the aqueous layer was discarded. The chloroform layer was washed twice with an equal volume of 2 M hydrochloric acid and twice with an equal volume of water. Subsequently, the chloroform was evaporated under a gentle stream of nitrogen at ambient temperature. The residue was dissolved in 500 μl of the mobile phase and 10 μl were injected into the HPLC system. The stability of acrolein in plasma was investigated as follows: 2.5 ml of a 1 μg ml^{-1} acrolein solution was added to 2.5 ml of drug-free plasma and to 2.5 ml of water. The solutions were stored at 4°C and every 15 min a 500 μl sample was taken and analysed immediately for undegraded acrolein after derivatization to HQ followed by HPLC injection and fluorimetric detection.

The interference of sodium 2-mercaptoethanesulphonate (mesna) with the reaction of the acrolein, prepared in situ, and 3-aminophenol was studied by adding either an equimolar amount of 4-hydroperoxyIF and mesna to plasma or 4-hydroperoxyIF without mesna. These samples were immediately analysed for their 4-hydroxyIF content with the derivatization method. Half of the plasma samples were stored at −20°C for 1 day, after which the 4-hydroxyIF concentration was determined.

2.5. Stability of 4-hydroperoxyIF, 4-hydroxyIF, MF and HQ

4-HydroperoxyIF and MF stock solutions, both 50 μg ml^{-1}, in citric acid buffer were stored at room temperature, 4 and −20°C. The samples were analysed by means of the described method. The stability of 4-hydroxyIF in patients' plasma and urine samples diluted 1:1 with a citric acid buffer pH 3.5 was tested after freezing 500 μl aliquots of the plasma and urine samples at −20°C and analysing two of these samples every day by means of the above-described method.

The stability of HQ, generated after derivatization of 4-hydroperoxyIF in plasma (2.8 μg ml^{-1}),

was tested at room temperature, 4 and −20°C. The samples from the freezer and refrigerator were analysed every day. The samples that were kept at room temperature were analysed every hour.

2.6. Patient's samples

Three plasma samples from a 7-year-old patient treated for a medulla blastoma with a 2 days of continuous IF infusion of 2.0 g m^{-2} on day 1 and 4.0 g m^{-2} on day 2 were analysed. The samples were taken before and at 4, 24, 28 and 48 h after the start of the infusion.

The plasma samples were collected into heparinized tubes containing 2.00 ml of citric acid buffer. Exactly 4.00 ml of the blood samples was added. The blood cells were then separated by centrifugation and the supernatant (a mixture of citric acid buffer and plasma) was stored immediately at −20°C until analysis.

Aliquots of 2.00 ml of urine samples were added to 2.00 ml of the stabilizing buffer solution, mixed and stored immediately at −20°C.

Thawing of the samples directly prior to analysis was done at ambient temperature.

3. Results and discussion

4-HydroxyIF is very unstable and is not commercially available. However, the peroxy compound 4-hydroperoxyIF is more stable and available. In many studies, the conversion of 4-hydroperoxyIF into 4-hydroxyIF by means of reduction with triphenylphosphine in dichloromethane at 0°C is utilized to obtain 4-hydroxyIF. These studies all refer to a paper by Takamizawa et al. [26]. In order to study the characteristics of 4-hydroxyIF during storage and derivatization, attempts were made to prepare 4-hydroxyIF, starting from 4-hydroperoxyIF, and by the reported reduction method [26]. The reaction was followed by HPLC with UV detection at 200 nm performed in a room at 4°C. However, so many peaks evolved that it was not possible to isolate the 4-hydroxyIF peak. Therefore, it was decided to study the recovery, accuracy and inter- and intra-day variation with 4-hydroperoxyIF and mafosfamide. According to various kinetic and metabolism studies [3,21,26–29], 4-hydroperoxyIF serves as a precursor to give 4-hydroxyIF, which is generated very quickly in biological and aqueous fluids without enzymatic involvement. Standard samples were also prepared from the cyclohexylamine salt of MF. It appears that the heating procedure results in complete hydrolysis of MF, thus releasing 4-hydroxyCP and mesna. 4-hydroxyCP was further hydrolysed, releasing acrolein that could be derivatized similar to ifosfamide-derived acrolein [19]. Consequently, MF can also be used for the preparation of calibration samples.

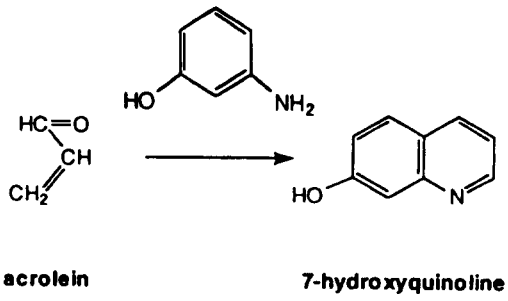


Fig. 3. Derivatization reaction of acrolein with 3-aminophenol.

3.1. Derivatization method and chromatography

The derivatization reaction is shown in Fig. 3.

The excitation optimum of HQ was established at 350 nm, whereas the maximum emission was measured at 510 nm. The optimal conditions for derivatization were 20 min at 100°C.

A chromatogram of HQ, after derivatization of a patient's plasma system, is shown in Fig. 4(a). HQ has a retention time of about 8 min in the described system. Without the addition of TMABr, the analyte elutes as a tailing peak. Addition of TMABr resulted in a symmetrical peak. Interfering endogenous plasma compounds were observed when the percentage of modifier in the mobile phase was increased. In some patients' samples a small peak at the same retention time as HQ was observed in the drug-free

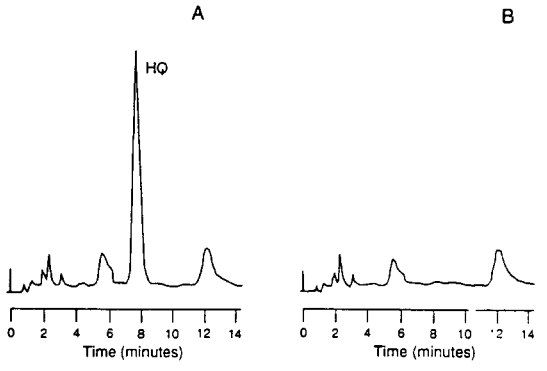


Fig. 4. HPLC trace after derivatization of a patient's plasma sample containing 0.98 μg ml^{-1} 4-hydroxyIF (A) and a drug-free sample (B). HQ = 7-hydroxyquinoline.

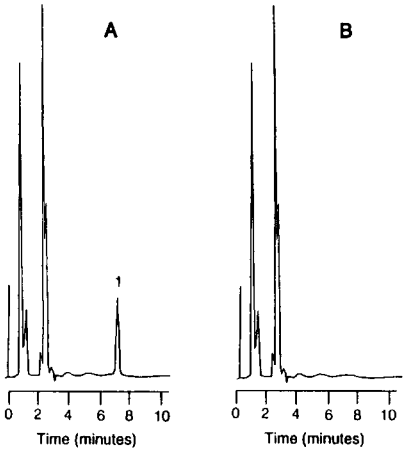


Fig. 5. HPLC trace of a spiked and derivatized (DNPH) plasma sample containing 100 ng ml^{-1} acrolein (A) and an acrolein-free sample (B). 1 = acrolein–DNPH derivative.

sample, probably due to the formation of HQ from acrolein, released from endogenous substances by the derivatization procedure. The concentrations were maximally equivalent to 0.1 μM 4-hydroxyIF. If necessary, the HQ peak heights of patients' samples were corrected by the individual blanks for each patient.

To evaluate the recovery of the fluorescent product under the conditions described, the fluorescence intensities of reaction mixtures initially containing various amounts of 4-hydroperoxyIF and MF were compared with those of HQ in standard solutions. Both compounds yielded ca. 60% of the fluorescent product, whereas an equimolar amount of acrolein also had a recovery of 60% HQ. Obviously, 4-hydroperoxyIF and MF are completely transformed into acrolein under these circumstances, but acrolein itself does not react quantitatively to HQ.

3.2. Validation

Calibration graphs were prepared in the concentration range 10–7000 ng 4-hydroxyperoxyIF/ml plasma and urine. The HQ peak heights versus concentration graphs were linear over the whole concentration range. The limit of detection in plasma and urine was 10 ng ml^{-1}.

The resulting calibration parameters are summarized in Table 1. The accuracy was tested by analysing five plasma or urine samples with a known 4-hydroperoxyIF concentrations. The derivatization recovery of 4-hydroperoxyIF was calculated by comparing the peak areas of the resulting HQ with a calibration graph of HQ in acid solution.

Unfortunately, acrolein cannot be used for the calibration graph because it binds to plasma

Table 1
Validation parameters for the determination of 4-hydroxyifosfamide in plasma and urine ($n = 5$)[a]

Matrix	Concentration (μg ml^{-1})	Recovery (%)	Accuracy (%)	Inter-day precision (%)	Intra-day precision (%)	Limit of detection (ng ml^{-1})
Plasma	0.1	55.2 ± 4.2	95.4	5.5	6.5	10
	2.0	57.6 ± 2.9	97.8	4.3	5.0	
Urine	0.1	59.3 ± 5.1	103.7	5.5	6.0	10
	2.0	58.2 ± 3.5	98.7	4.5	5.2	

[a]plasma: $y = 0.95(\pm 0.02) + 0.41(\pm 0.01)x$ ($r = 0.9989$). Urine: $y = 0.41(\pm 0.09) + 0.79(\pm 0.04)x$ ($r = 0.9990$).

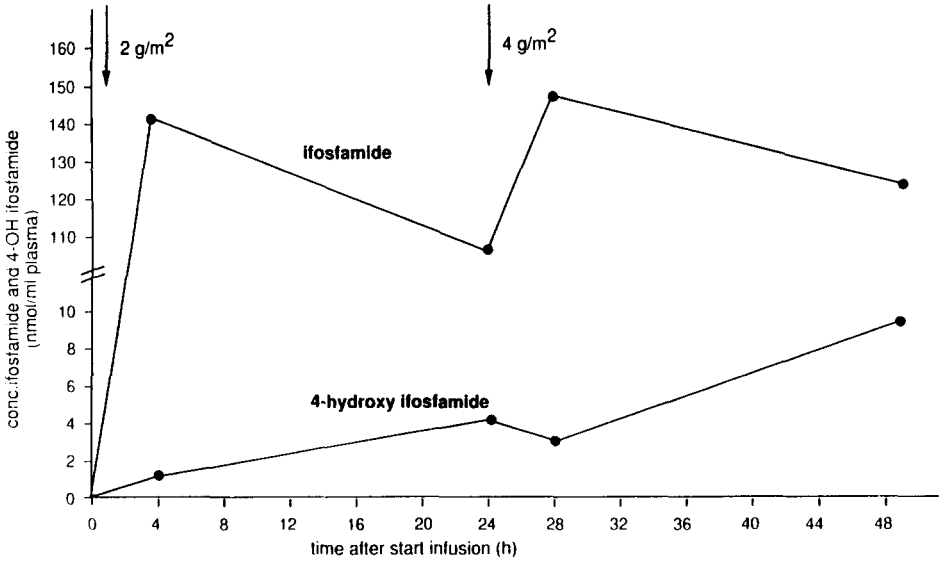


Fig. 6. Plasma concentration–time curve for IF and 4-hydroxyIF in a sample found in a patient receiving a continuous infusion of IF for 2 days (2 g m^{-2} on day 1 and 4 g m^{-2} on day 2).

proteins within minutes when added to plasma. Apparently, this phenomenon does not take place when acrolein is formed from 4-hydroperoxyIF or 4-hydroxyIF in the presence of 3-aminophenol.

When acrolein is added to plasma, only 10% of the initial concentration remains after 20 min. No acrolein could be detected after 60 min. Since no decrease in acrolein concentration could be observed in aqueous solutions of the compound, adsorption on the container walls does not occur. Acrolein is a very reactive compound and it is unlikely that unbound acrolein will be detected in patients' plasma [25,30,31]. Patients' plasma samples were analyzed 1 day after withdrawl by a derivization method not affecting 4-hydroxyIF [25]. A chromatogram of the DNPH–acrolein derivative in plasma is shown in Fig. 5(a). No acrolein could be detected in the patient's plasma or urine samples whereas acrolein added to drug-free plasma or urine after immediate analysis was detectable to a minimum concentration of 20 ng ml^{-1}. Acrolein below this concentration would result in a maximum concentration of 100 ng ml^{-1} falsely attributed to a 4-hydroxyIF. However, the fast disappearance rate of acrolein when added to plasma makes it very unlikely that high concentrations of acrolein will be present in the plasma, ruling out the interference of acrolein for the determination of 4-hydroxyIF [32]. As expected, no free acrolein was detected in urine: the concommitant administration of mesna prevents the formation of acrolein from 4-hydroxyIF and the already existing acrolein is transformed into a mesna adduct. The interference of mesna with the derivization reaction of 4-hydroxyIF was found to be non-existent.

Lidocaine-HCl was added to the stabilizing buffer solutions for an accurate calculation of the dilution of the plasma samples. Lidocaine was determined simultaneously with IF, 2- and 3-dechloroethylIF by gas chromatography, as described earlier [33,34], and was compared with the lidocaine concentration in the buffer which was not diluted with plasma or urine and stored under exactly the same conditions as the plasma and urine samples.

3.3. Stability

Sladek et al. [29] found the half-life of 4-hydroxyCP, after addition to plasma, to be ca. 6 min, whereas it is ca. 12 min in 50% serum at

37°C. The disappearance of 4-hydroxyCP paralleled the appearance of phosphoramide mustard. The rate of conversion is markedly pH and temperature dependent. The conversion decreased when acidified serum was used: the half-life increased to more than 100 min. The apparent stability reported for 4-hydroxyCP in Tris buffer results from the formation of a stable Tris–4-hydroxyCP adduct, indicating that Tris is not an effective compound for stabilizing samples.

The rate-limiting step in the drug activation involves β-elimination of PM from aldoifosfamide, presumably via general base catalysis, which is characteristic of eliminations involving weakly basic leaving groups [35]. Kurowski et al. [19] added ethyl acetoacetate to plasma samples in order to stabilize 4-hydroxyIF by an excess of carbonyl compounds. In the present study, the stabilization of 4-hydroxyIF after acidification of plasma samples with citrate buffer (pH 3.5), according to Ikeuchi et al. [16], proved to be satisfactory. When patients' plasma samples are stored at −20°C, sample clean-up and derivatization have to be performed within 3 days in order to limit the degradation of 4-hydroxyIF.

The t_{95} (95% remaining concentration) of 4-hydroperoxyIF in the stock solution is 2.5 h at room temperature, 13 h at 4°C and 4 days at −20°C. Consequently, the 4-hydroperoxyIF stock solution has to be renewed every 2 days. A fresh derivatizing reagent solution (3-aminophenol–hydroxylamine) has to be prepared every 3 days [18].

HQ, prepared from 4-hydroperoxyIF, in plasma can be kept in a refrigerator (4°C) for at least 8 days. Hence, the samples do not have to be analysed immediately after the derivatization reaction.

In order to be able to determine as many IF metabolites as possible in one sample, the stability of IF and its two dechloroethylated metabolites was also established. The stability of IF and 3-dechloroethylIF in citrate buffer when stored at −20°C appeared to be good: only 5% of the compounds had degraded after 60 and 23 days, respectively. 2-DechloroethylIF could not be reliably determined in plasma and urine samples diluted with citrate buffer; after 24 h 50% of the 2-dechloroethylIF had disappeared. However, 2-dechloroethylIF is known to be stable in ethyl acetate solution [34]. Therefore, an immediate clean-up of the samples, as described for the GC determination of IF and 2- and 3-dechloroethylIF, is advised in order to determine 2-dechloroethylIF.

3.4. Patient's sample

The plasma concentration–time curve of IF and 4-hydroxyIF of a patient's sample is shown in Fig. 6. The plasma concentrations of 4-hydroxyIF did not exceed 10 μM.

4. Conclusions

In comparison with the direct fluorimetric quantitation of acrolein-derived HQ [10], the HPLC method presented here has improved the accuracy and sensitivity of the assay manyfold. Plasma and urine samples appeared to be stable for 3 days when stabilized at pH 3.5. IF and 3-dechloroethylIF are also stable at pH 3.5 and can be determined by a GC method [33,34]. The method presented has proven its suitability for the determination of 4-hydroxyIF in patients' plasma and urine samples and is currently in use for pharmacokinetic studies.

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