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Determination of chloroacetaldehyde, a metabolite of oxazaphosphorine cytostatic drugs, in plasma

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ABSTRACT

A derivatization high-performance liquid chromatographic method with ultraviolet detection to monitor the plasma concentration of chloroacetaldehyde, a neurotoxic metabolite of oxazaphosphorine drugs, is presented. To prevent the rapid degradation of chloroacetaldehyde, the plasma samples are stabilized with formaldehyde. The method is linear in the concentration range 1–250 nmol/ml. Blood samples from a patient who was treated with a ten-day continuous infusion of ifosfamide were assayed. The chloroacetaldehyde concentrations did not exceed 10 nmol/ml.

INTRODUCTION

Ifosfamide (IF), a representative of the oxazaphosphorine alkylating agents, is widely used in the treatment of various forms of cancer [1]. This family of anticancer drugs requires metabolic activation to a phopshoramide mustard to exert cytotoxicity. Concurrent deactivation reactions generate the inactive metabolites 2- and 3-dechloroethylifosfamide and chloroacetaldehyde (CA)

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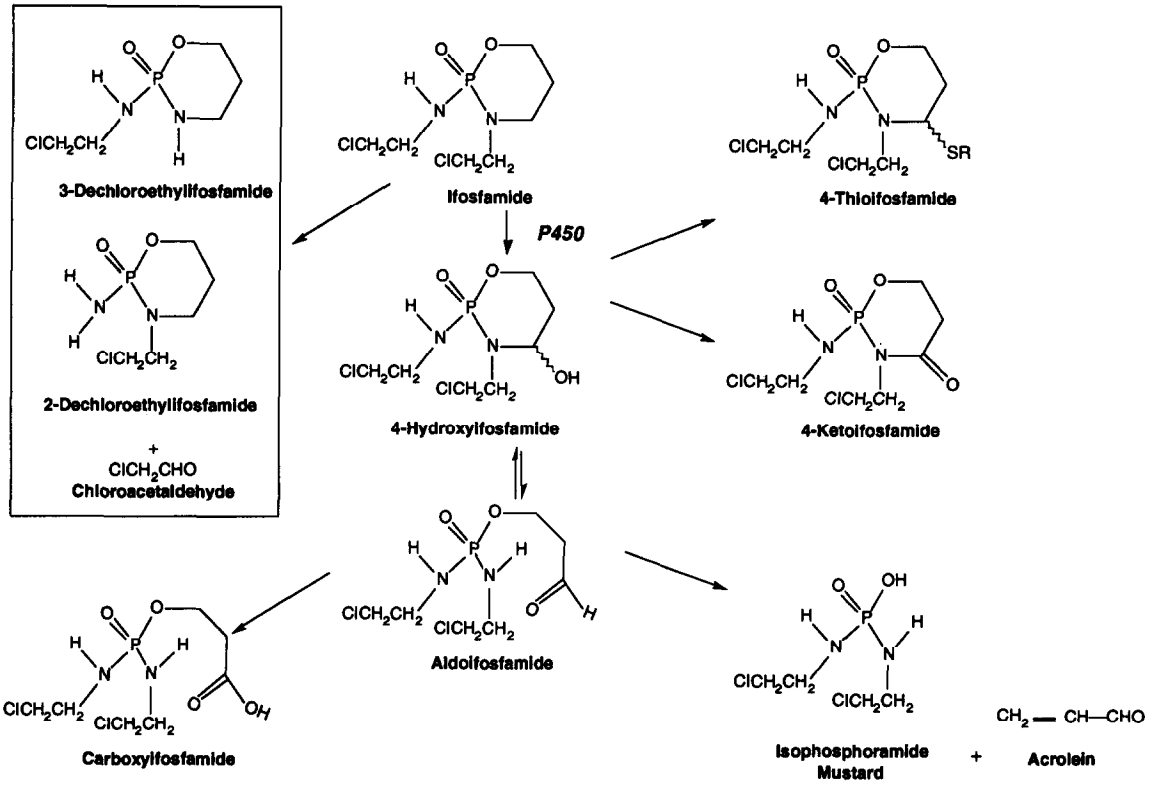

Fig. 1. Metabolism of ifosfamide.

[2]. Fig. 1 shows the metabolic activation and inactivation reactions of IF. During IF therapy some patients may develop serious neurotoxic adverse effects, which have been related to the formation of the metabolite CA [3–5]. It is clear that CA monitoring during IF therapy might extend the insight into the assumed relation between CA levels and drug-induced neurotoxicity. Several techniques have been described for the analysis of CA, mostly based on derivatization. Derivatization of CA with 2,4-dinitrophenylhydrazine in combination with thin-layer chromatography (TLC) of the resulting hydrazone has been utilized to quantify radiolabelled CA [6]. After derivatization of CA with 2-hydrazinobenzothiazole [7] or 3-methyl-2-benzothiazolonehydrazone [8], the reaction product can be analysed colorimetrically. A fluorimetric assay of CA has been reported after derivatization of CA with cyclic AMP with the formation of $1,N_6$-ethenoadenine [9]. The derivatization product of CA with thiourea can be chromatographed by means of a high-performance liquid chromatographic (HPLC) system with UV detection [10]. Underivatized CA has been analysed by means of gas chromatography (GC) [3,11–13] with flame ionization [11] or electron-capture detection [3,12].

A major drawback of these techniques is that they have not been developed for the analysis of CA in biological fluids such as plasma and urine, with the exception of the TLC assay, however, this method lacks the detection limit that is required. Ruzicka and Ruenitz [10] have quantified CA in microsomal preparations after derivatization with thiourea.

This report describes an assay for the analysis of CA in the plasma of patients treated with IF. The method is based on the conversion of CA in plasma with thiourea into 2-aminothiazole,

which is assayed by HPLC after solid-phase extraction (SPE). Special attention is paid to the stability of the analytes.

EXPERIMENTAL

Chemicals

CA, as a 45% (v/v) solution in water, and thiourea were purchased from Merck (Darmstadt, Germany). The exact concentration of CA was established by an oxim titration [14]. 2-Aminothiazole was obtained from Janssen Pharmaceutica (Geel, Belgium). The chemicals were used as received.

Analytical reagent-grade methanol, originating from Westburg (Leusden, Netherlands), was used. All other chemicals were of analytical grade and used as received. Distilled water was used throughout the study.

Instrumentation

The HPLC system consisted of a Model 510 pump, a U6K injector and a Model 440 detector (all from Waters, Milford, MA, USA) operating at a wavelength of 254 nm. Chromatography was performed with a ChromSpher C_8 column (150 mm × 4.6 mm I.D., 5 μm particle size, Chrompack, Middelburg, Netherlands). The mobile phase was a mixture of methanol and 10 m*M* phosphate buffer solution with 0.05% triethylamine pH 7.4 (5:95, v/v) used at a flow-rate of 1.0 ml/min. The mobile phase was passed through a 0.2-μm filter and degassed by sonication prior to use. Peak areas were measured with an SP4270 integrator (Spectra Physics, San José, CA, USA).

Plasma clean-up procedure and derivatization

Before analysis CA was derivatized with thiourea in the plasma matrix (Fig. 2). A 500-μl volume of plasma was deproteinized with 60 μl of perchloric acid (70%). After centrifugation (3000 *g* for 2 min) 200 μl of the supernatant were transferred to a 0.8-ml crimp-capped vial (Chromacol, London, UK) and 30 μl of a thiourea solution (760 mg per 100 ml) were added. The mixture was heated at 90°C for 2 h in a thermostatically controlled waterbath at 90°C.

After cooling the samples to room temperature, the derivatization product, 2-aminothiazole, was isolated by SPE. SPE was carried out with cation-exchange sorbent-containing columns (1 ml Bond Elut SCX, Analytichem, Harbor City, CA, USA). The columns were preconditioned by washing with 1 ml of methanol, 1 ml of acetonitrile, 2 × 1 ml of water and 2 × 1 ml of 1% acetic acid under reduced pressure (Vac-Elut, Analytichem). The sorbent was not allowed to dry. After this procedure the whole sample volume was applied onto the column. The column was then washed four times with 1 ml of 1% (v/v) acetic acid and three times with 1 ml of 1% (v/v) acetic acid–methanol (50:50, v/v). Finally, the 2-aminothiazole reaction product was eluted with 1 ml of a mixture of methanol and 14 *M* ammonia (96:4, v/v). Next, 60 μl of a 3 *M* hydrochloric acid solution were added to the eluate to achieve a pH of about 6. The sample was concentrated by evaporating the solvents under a stream of nitrogen at 35°C. The dry residue was dissolved in 200 μl of the mobile phase. A 20-μl aliquot was injected into the HPLC system.

Chemical stability

The stability of the stock solution of CA (60 μ*M*) was tested by taking a sample every week. The samples were treated as described above.

In order to prevent disappearance of CA, the plasma samples were stabilized with formaldehyde. To 5.0 ml of heparinized whole blood, 100 μl of a 50 m*M* formaldehyde solution were add-

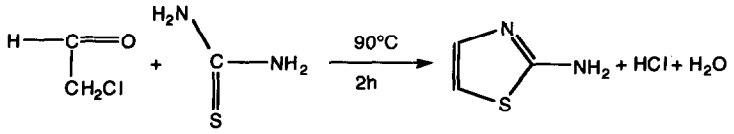

Fig. 2. Derivatization reaction of chloroacetaldehyde with thiourea.

ed. The blood samples were centrifuged immediately and the resulting supernatant was stored at −20°C for stability testing.

The stability of CA in whole blood without the addition of a stabilizing agent was determined by spiking 5.0 ml of blood with 50 μl of a 2 m*M* CA solution (final concentration: 100 nmol of CA per ml of blood). The mixture was kept in the refrigerator and a sample was taken every 5 min. The samples were treated in the same way as plasma samples; deproteinization of the samples also caused a precipitation of the blood cells, yielding a clear fraction.

The stability of CA in plasma (100 nmol/ml) was determined at three different temperatures: room temperature, 4°C and −20°C. The plasma stored in the refrigerator and the freezer was sampled every 24 h, while the plasma kept at room temperature was sampled every 30 min. The samples were stored in 1.5-ml portions. The CA analyses, at each time point, were performed in duplicate.

Plasma samples spiked with CA were processed as described in the *Plasma clean-up procedure and derivatization* section. The resulting solutions, in mobile phase, of 2-aminothiazole, the end-product of the derivatization reaction, were immediately analysed. The remaining solutions were stored at 4°C and analysed every day for a maximum of seven days.

Patient samples

A 68-year-old patient with advanced colon cancer, previously untreated, received 2.5 g IF per day by continuous infusion over ten days, and was monitored for CA using the described method. Plasma concentrations of IF, 2- and 3-dechloroethylifosfamide were analysed by a capillary GC method [15].

RESULTS AND DISCUSSION

Plasma clean-up procedure and derivatization

Deproteinization of the plasma samples before the derivatization reaction is necessary. Various procedures were tested and recoveries of CA were assessed. Deproteinization with perchloric acid gave a recovery of approximately 75%, while acetonitrile or a mixture of zinc sulphate and barium hydroxide yielded recoveries of less than 40%. Ruzicka and Ruenitz [10] used a mixture of zinc sulphate and barium hydroxide to deproteinize microsomal samples containing CA with recoveries of less than 40%. It appears that these low recoveries are mainly due to the deproteinization procedure. A disadvantage of the use of perchloric acid could be that the resulting supernatant has a very low pH (*ca.* 2), which might negatively influence the derivatization reaction, but this was not the case.

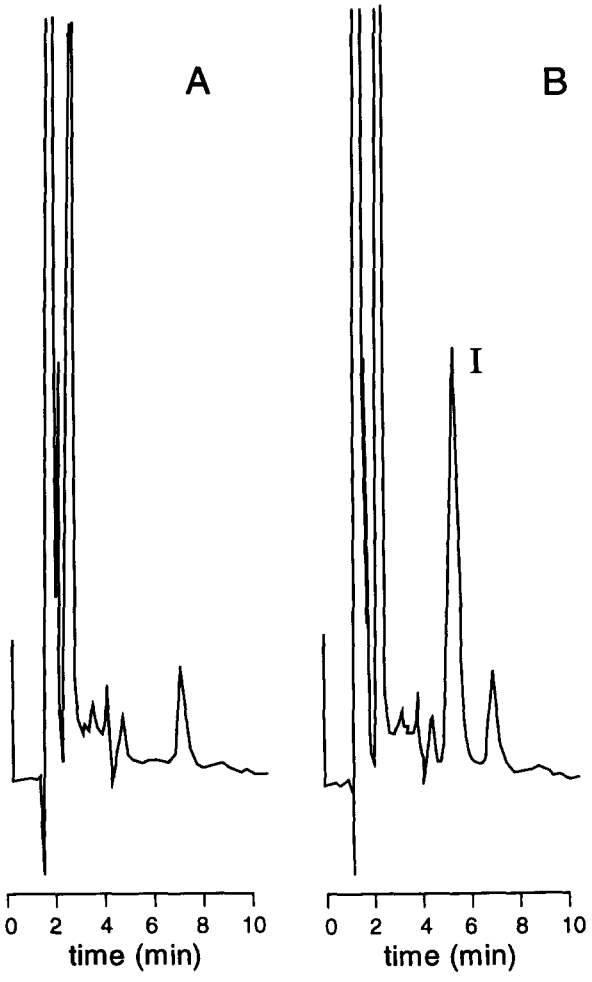

Fig. 3. Chromatogram, after derivatization, of a CA-free plasma sample (A) and a patient sample containing 5 nmol/ml CA (B). I = 2-aminothiazole.

TABLE I

VALIDATION PARAMETERS OF THE DETERMINATION OF CA IN PLASMA (n = 5)

Concentration (nmol/ml)	Recovery (%)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
50	78.3	103.4	5.2	5.5
100	79.7	102.8	4.9	5.0
150	77.6	98.9	4.8	5.0
200	83.6	103.2	4.9	5.0
250	79.1	97.2	4.1	4.8

The optimum temperature for the derivatization reaction was established as 90°C. After 2 h at this temperature the 2-aminothiazole concentration reached a maximum.

After the SPE procedure the pH of the eluate was adjusted to 6. At this pH, 2-aminothiazole is converted to its chloride salt, making it less volatile.

Chromatography and detection

Fig. 3 shows a chromatogram of a CA-free and a patient plasma sample after the derivatization procedure. The UV absorbance of 2-aminothiazole was measured at the wavelength of maximum absorbance at 254 nm.

Formaldehyde also reacts with thiourea. The end-product of this reaction is N-(hydroxymethyl)thiourea, which may condense to resin-like components [16]. In the chromatogram, however, no peak of the derivatization product of formaldehyde was noticed.

Performing the derivatization reaction with acrolein (2-propenal), another metabolite of oxazaphosphorine drugs, does not lead to a detectable product in the above-described system.

Validation

Calibration graphs were made ranging from 0 to 250 nmol CA per ml of plasma with correlation coefficients >0.999.

The percentage recoveries of known quantities of CA in plasma were calculated for various concentrations by measuring the absolute quantity of 2-aminothiazole recovered, using a calibration curve of 2-aminothiazole dissolved in the mobile phase.

The recoveries, accuracy, intra- and inter-assay precision were determined at various CA concentrations and are summarized in Table I. The intra-assay precision was measured for five samples on the same day, and the inter-assay precision samples were quantified on five consecutive days. The limit of detection (signal-to-noise ratio of 3:1) is 0.5 nmol of CA per ml of plasma with an injection volume of 20 μl.

Chemical stability

All stock solutions of CA in water were stable for at least four months when stored at 4°C. Cerny and Küpfer [13] reported a half-life ranging from 2 to 6 min for CA in whole blood at ambient temperature. These investigators proposed the addition of formaldehyde (40 nmol/ml) to freshly collected blood samples to prevent CA decline. The half-life of CA in whole blood found in this study (30 min) was higher than determined by Cerny and Küpfer [13]. The half-lives of CA in plasma at room temperature, 4°C and −20°C were 4.2 h, 82 h and 7 days, respectively. The very rapid decline of CA in blood and plasma warrants the use of a stabilization procedure. A 100-μl aliquot of a 50 mM formaldehyde solution very effectively stabilized the plasma samples with the advantage that a small volume of 100 μl does not cause a considerable dilution of the sample. A final concentration of 1 μmol of formaldehyde per ml of blood was sufficient to stabilize CA in plasma stored at −20°C for at least two weeks with no chromatographic interferences.

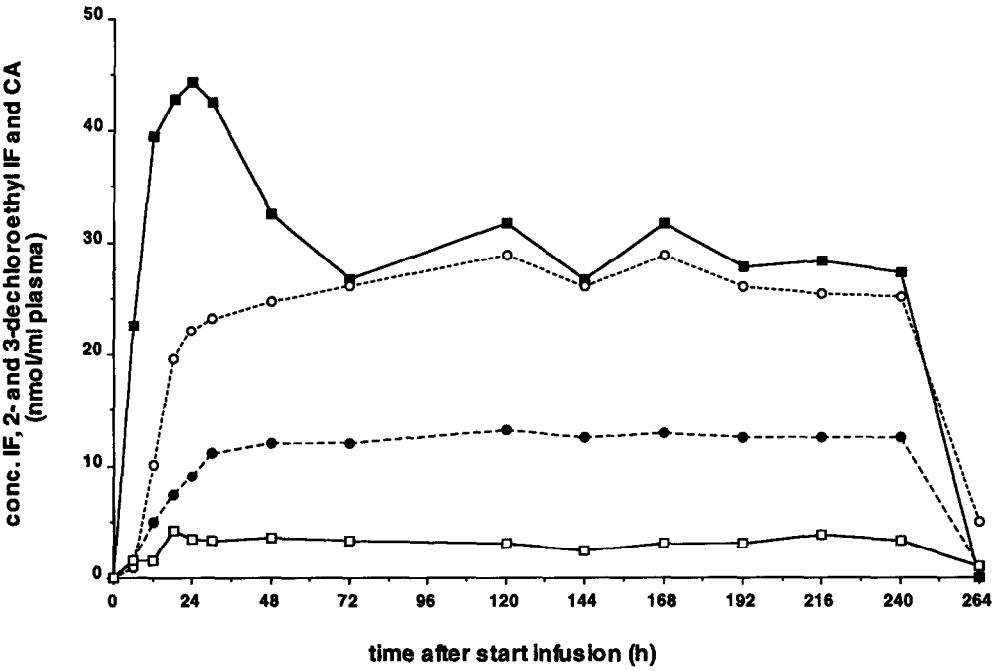

Fig. 4. Plasma concentrations of IF (■), 2-dechloroethylifosfamide (◆), 3-dechloroethylfosfamide (○) and CA (□) in a patient receiving a ten-day continuous ifosfamide infusion of 2.5 g per day.

The end-product of the derivatization reaction, 2-aminothiazole, dissolved in mobile phase and stored in the refrigerator (4°C), was stable for at least one week.

Patient samples

The results of the analysis of patient samples are presented in Fig. 4. In the described patient, CA concentrations of up to 6.4 nmol/ml (0.5 μg/ml) were found without symptoms of neurotoxicity. These concentrations, however, are well below the previously reported toxic CA concentration of 80–100 nmol/ml [3]. So far, only plasma samples form patients treated with a continuous ten-day infusion have been analysed. None of them showed any serious neurotoxic side-effects, although a number of these patients complained of drowsiness and a decreased ability to concentrate. These low plasma values compared with the values of up to 200 nmol/ml found by Cerny and Küpfer [13] may be explained by different dosage schedules. Continuous infusion of IF rather than short bolus infusions is known to reduce the risk of developing neurotoxic symptoms [17,18].

CONCLUSIONS

A simple, selective and sensitive method for the analysis of CA in patients receiving IF therapy is described. CA in blood and plasma needs to be stabilized by adding a formaldehyde solution directly to the blood samples.

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