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Determination of 4-hydroxycyclophosphamide in plasma, as 2,4-dinitrophenylhydrazone derivative of aldophosphamide, by liquid chromatography

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Abstract

A reversed-phase liquid chromatographic method to determine the concentration of 4-hydroxycyclophosphamide, a labile cytotoxic metabolite of cyclophosphamide, in plasma is described. In order to stabilize 4-hydroxycyclophosphamide, as well as to increase the selectivity and the sensitivity, a 2,4-dinitrophenylhydrazone derivative was formed. Plasma proteins were precipitated with acetonitrile prior to the derivatization with 2,4-dinitrophenylhydrazine at pH 2. The derivatization was performed at 50°C for 5 min. The chromatographic system consisted of an octadecyl silica column and a mobile phase containing phosphate buffer and acetonitrile. Quantitation was performed using an UV detector operating at 357 nm. Optimal derivatization was obtained by adding 0.2 ml 2,4-dinitrophenylhydrazine (3.8 mg/ml) to 0.5 ml of the deproteinized plasma supernatant. The relative recovery of 4-hydroxycyclophosphamide from plasma is $\geq 97\%$. Concentration levels down to 22 ng/ml of 4-hydroxycyclophosphamide in plasma could be determined with a R.S.D. of about 13%. No degradation of the derivative was observed after 24 h at room temperature. The $t_{1/2}$ for 4-hydroxycyclophosphamide in blood is ca. 4 min at 37°C, whereas 4-hydroxycyclophosphamide is stable for at least 1 h at 4°C. Application of the method for the pharmacokinetic monitoring of 4-hydroxycyclophosphamide is described.

1. Introduction

Cyclophosphamide (CP) is one of the most useful anticancer and immunosuppressive agents [1]. CP acts as a prodrug since it is inactive until it undergoes hepatic transformation to form 4-hydroxycyclophosphamide (4OHCP). 4OHCP has a tautomeric form, aldophosphamide, which generates phosphoramide mustard (PM) and acrolein by β-elimination [2]. PM is considered to be the ultimate alkylating agent, acting in the cells [1,2]. However, PM can not move in and out the cells as freely as 4OHCP and its tautomeric form can, therefore it is important to determine the extracellular plasma concentration of 4OHCP/aldophosphamide. 4OHCP is very unstable and has low molar absorption and only a few methods for determination of the concentration in plasma are available [3–5]. 4OHCP has been determined indirectly by a fluorimetric assay [3], by TLC with a radioassay [4] and by using GC–MS and a stable isotope as internal standard [5].

In the present study 4OHCP has been con-

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verted into aldophosphamide as the sample was acidified (the ring-opening reaction of 4OHCP is acid-catalysed [6]). The aldo form has been stabilized by 2,4-dinitrophenylhydrazine and the derivative has been chromatographed by liquid chromatography on a reversed-phase octadecyl column and detected with UV at 357 nm.

4OHCP has a chiral phosphorus atom and exists in two enantiomeric forms. These two forms have been separated on a Chiral-AGP column as their 2,4-dinitrophenylhydrazone derivatives.

2. Experimental

2.1. Chemicals

4-Hydroperoxycyclophosphamide (4OHOCP), which generates rapidly 4-hydroxycyclophosphamide in aqueous solution, was kindly supplied by Dr. J. Pohl Asta Medica, Frankfurt am Main, Germany.

2,4-Dinitrophenylhydrazine (analytical grade) was obtained from E. Merck, Darmstadt, Germany. All other chemicals and solvents used were of HPLC or analytical grade.

2.2. Apparatus and chromatography

The liquid chromatographic system consisted of an LKB Model 2150 pump, Pharmacia Biotech Norden, Sollentuna, Sweden, an automatic injector, CMA 200, CMA Microdialysis, Stockholm, Sweden, and a Shimadzu SPD 10 A UV detector from Polynom, Sollentuna, Sweden, operating at 357 nm. For peak integration a PE Nelson Turbochrom version 3.3 chromatographic system, Perkin-Elmer, Norwalk, CT, USA, was used.

The analytical column was a Spherisorb S3ODS2 (100 × 4.6 mm I.D., 3 μm) and the precolumn was a Spherisorb S3ODS2 (10 × 3.2 mm I.D., 3 μm); both columns were obtained from Hichrom, Berkshire, UK. The mobile phase was a phosphate buffer pH 7 (ionic strength, $\mu = 0.02$) containing 40% (v/v) acetonitrile. The flow-rate was 1.0 ml/min.

For the semi-preparative isolation of 4OHCP 2,4-dinitrophenylhydrazone derivative, a Brownlee OD257 Spheri-5 (250 × 7 mm I.D., 5 μm) column from Brownlee Labs., CA, USA, was used. The mobile phase consisted of water–acetonitrile (50:50). The same apparatus as the analytical system was used except for the detector which was an LKB 2151 variable-wavelength UV detector from Pharmacia Biotech Norden, working at 357 nm. The detector cell volume was 10 μl. The flow-rate was 4.0 ml/min.

The chiral separation was obtained on a Chiral-AGP column (100 × 4.0 mm I.D. 5 μm) obtained from ChromTech, Hägersten, Sweden. The mobile phase was a phosphate buffer pH 7.2 ($\mu = 0.02$) containing 5% (v/v) acetonitrile. The flow-rate was 1.0 ml/min. The same apparatus as the in the analytical system was used for chromatography and detection.

2.3. Derivatization

In order to prepare a batch of 4OHCP 2,4-dinitrophenylhydrazone derivative, 20 mg of 4OHOCP were dissolved in 15 ml water. To this solution, 15 ml of 2,4-dinitrophenylhydrazine (9.1 mg/ml) and 1.05 ml of 1 *M* hydrochloric acid were added. The mixture was shaken on a vortex and heated for 5 min at 50°C. Sample volumes of 1000 μl were injected onto the semi-preparative chromatographic system described above. At the outlet of the detector the peak containing the 4OHCP 2,4-dinitrophenylhydrazone derivative was collected. The acetonitrile in the sample was evaporated under a gentle stream of nitrogen. In pure water, the crystals of the derivative precipitated. The crystals were filtered, washed with water and dried in vacuum. These crystals were used to identify and to determine the purity of the derivative by LC–MS and NMR (see below under *Derivative identification*).

2.4. Sample handling

Blood samples from patients were collected in cold EDTA tubes and placed in an ice bath. Within 2 min of collection, the tubes were

centrifuged for 10 min at 2000 *g* (4–8°C). Plasma fractions (0.50 ml) were transferred to new cold tubes and 1.00 ml cold acetonitrile was added to each of them. The tubes were shaken for 30 s and centrifuged for 3 min at 6000 *g*. The supernatants were transferred to eppendorf tubes and stored at −70°C.

2.5. Sample preparation and derivatization

To 1.00 ml supernatant, 70 μl of 1 *M* hydrochloric acid and 200 μl of 2,4-dinitrophenylhydrazine (3.8 mg/ml acetonitrile) were added. The mixture was shaken on a vortex for 30 s and heated for 5 min at 50°C. A 40-μl volume of the solution was injected onto the chromatographic system. The 2,4-dinitrophenylhydrazine solution was prepared new every second week.

2.6. Calibration curves, within-day and between-days precision and relative recovery

The standard samples used for the calibration curves were prepared as follows: to 0.50 ml plasma, 30 μl of 4OHCP standard solution was added. To precipitate the plasma proteins, 1.00 ml acetonitrile was added and the mixture was shaken on a vortex for 30 s and centrifuged for 3 min at 6000 *g*. Derivatization was performed as described above. The peak areas were plotted versus the concentration of 4OHCP added to plasma. The calibration curve range was between 22–2180 ng/ml.

The within-day precision of the method was examined at four different concentrations of 4OHCP (22, 33, 272 and 1090 ng/ml plasma). The samples were handled as described under *Sample preparation and derivatization* and the relative standard deviations were calculated.

In order to determine the between-days variation, plasma samples deproteinized with acetonitrile were spiked with 4OHCP at a concentration of 813 ng/ml. Fractions of the samples were frozen at −70°C and analyzed each day when the patient samples were analyzed. The relative recovery for 4OHCP was obtained by comparing the peak areas obtained after derivatization of 4OHCP in plasma with peak areas obtained after derivatization of 4OHCP in water.

2.7. Stability

Stability of 4OHCP in whole blood at 37 and 4°C

A 20-ml volume of fresh whole blood (37°C) was spiked with 288 μg of 4OHCP. A 10-ml volume of the spiked blood sample was placed in a tube in an ice bath (4°C) while the remaining 10 ml were kept at 37°C. After 5, 10, 20, 40 and 60 min aliquots of blood samples (1.5 ml) were taken from both tubes. (The samples from the warm blood were chilled in the ice bath for 30 s). Each aliquot was than centrifuged at 4°C for 5 min at 2000 *g* and 0.5 ml of the supernatant (plasma) was handled and derivatized as described above. A 20-μl volume of the derivatized samples was injected onto the chromatographic system.

Stability of 4OHCP in plasma and plasma deproteinized with acetonitrile at 25°C

A series of plasma samples (0.5 ml) were spiked with 3.4 μg of 4OHCP. A set of plasma samples were immediately deproteinized with 1.00 ml acetonitrile, as described above. The deproteinized plasma samples were stored for 5, 10, 20, 40 and 60 min ($n = 2$ per time point) before they were derivatized. Another set of plasma samples were stored for 0, 5, 10, 20, 40 and 60 min ($n = 2$ per time point) before they were deproteinized with 1.00 ml of acetonitrile and handled as described above. All samples were derivatized as described under *Sample preparation and derivatization*. A 20-μl volume of the samples was injected onto the chromatographic system.

Stability of 4OHCP in plasma, plasma deproteinized with acetonitrile and as 2,4 dinitrophenylhydrazone derivative at −70°C

A series of plasma samples (0.5 ml) were spiked with two different concentrations of 4OHCP, 51 and 813 ng/ml. A set of plasma samples with high and low concentrations of 4OHCP ($n = 8$ apiece) were immediately placed

at −70°C. Another set of samples at high and low concentrations of 4OHCP were deproteinized with 1.00 ml acetonitrile and the deproteinized supernatants were placed at −70°C. A third set of high and low concentration samples were handled as described under *Sample preparation and derivatization* and the derivatives were placed at −70°C. The stability of the three set of samples was determined in duplicate, after 1, 12, 25 and 100 days of storage at −70°C.

2.8. Derivative identification

In order to identify the 4OHCP 2,4-dinitrophenylhydrazone derivative a LC–MS spectrum was obtained on a TSQ 70 Finnigan MAT, with loop injection. The mobile phase was a 50 m*M* ammonium acetate buffer containing 30% methanol. The flow-rate was 1.5 ml/min. A deuterium NMR spectra of the derivative was obtained on a Jeol JNM-EX270 instrument. The 4OHCP 2,4-dinitrophenylhydrazone derivative sample was dissolved in deuterium-labeled acetonitrile and tetramethylsilane was used as the internal standard.

2.9. Subject

A female patient with a breast cancer received an intravenous bolus dose of cyclophosphamide (1100 mg). The patient also received 5-fluorouracil and epirubicine.

3. Results and discussion

CP undergoes hepatic transformation to 4OHCP which has a tautomeric form, aldophosphamide (Fig. 1). Both 4OHCP and aldophosphamide can be converted to inactive metabolites, 4-ketocyclophosphamide and carboxyphosphamide, respectively. However, β-elimination of the aldophosphamide gives acrolein and PM. PM is considered to be the ultimate alkylating agent acting in the cells [1,2]. As the transport of PM into the cells is negligible in comparison with the transport of the 4OHCP/aldophosphamide forms (below denominated as 4OHCP), the intracellular concentration of PM depends on the extracellular concentration of 4OHCP. It is very difficult to determine the intracellular plasma concentration of PM and therefore the extracellular plasma concentration of 4OHCP was determined in this study. 4OHCP is very labile and it is important to stabilize the compound as soon as possible after the plasma sample is taken. Sladek [7] used semicarbazide to prepare a stable semicarbazone derivative of 4OHCP. However, this derivative has a rather poor molar absorptivity (ϵ) in the UV range. In order to improve the UV absorptivity of 4OHCP the aldo form can be derivatized with 2,4-dinitrophenylhydrazine, which reacts easily with aldehydes, ketones and α-keto acids [8–10].

3.1. Derivatization

4OHCP has been stabilized by derivatization with 2,4-dinitrophenylhydrazine. The derivative has three UV maxima, at 212, 263 and 357 nm. The highest ϵ was obtained at 357 nm and that wavelength was used for the analysis. In order to optimize the derivatization conditions, the effects of pH and temperature were observed. In Table 1, it is demonstrated that pH 2 and 5 min at 50°C gives the optimum peak height. This is in agreement with the fact that the ring-opening reaction of 4OHCP (Fig. 1) is acid-catalysed [6] and that aldehydes react easily with 2,4-dinitrophenylhydrazine [9].

Fig. 2 demonstrates the effect of the 2,4-dinitrophenylhydrazine concentration on the peak area of the derivative. The concentration was increased from 0.9 to 12.2 mg/ml. It was not possible to dissolve 16 mg 2,4-dinitrophenylhydrazine in 1 ml acetonitrile as the solution was saturated. For the analysis a concentration of 3.8 mg/ml was used, this gives a molar excess of 2,4-dinitrophenylhydrazine over the 4OHCP (7.2 μg/ml plasma) of 400 times. In plasma samples the excess of the reagent over 4OHCP was approximately 10 000 times. It is necessary to have a large excess of the reagent, since plasma samples include many endogenous ketones and aldehydes which can react with the reagent. However, it was not possible to use a concen-

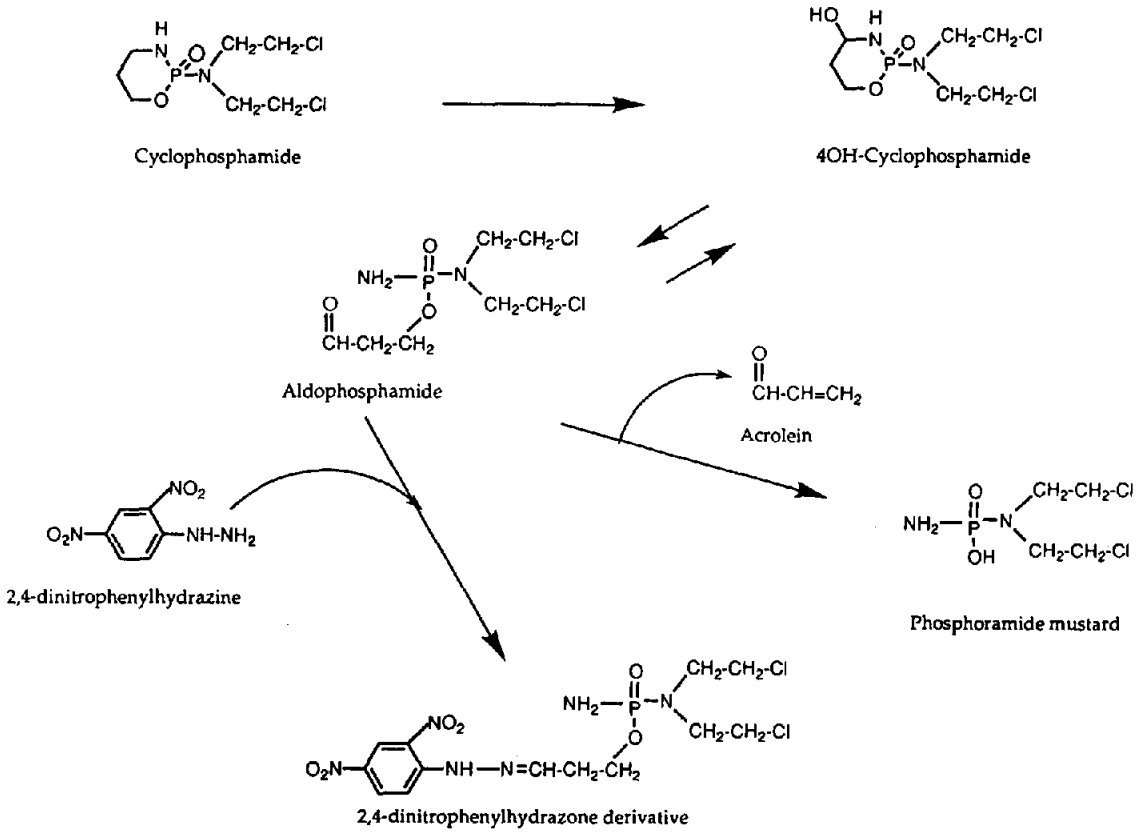

Fig. 1. Chemical structures of cyclophosphamide and its metabolites. Reaction scheme for the derivatization of 4-hydroxycyclophosphamide with 2,4-dinitrophenylhydrazine.

tration of the reagent greater than 3.8 mg/ml even though it is demonstrated in Fig. 2 that the peak area would increase, as the 4OHCP derivative peak was eluted on the slope of the excess of the reagent in the chromatographic system.

Table 1
Optimization of the derivatization conditions

pH	Time at 50°C (min)	Peak height (mm)
5	0	<2
2	0	4.5
2	5	37
2	10	36
2	30	31
2	60	24

Concentrations in the final solution were: 0.07 mg/ml of 4OHCP and 0.75 mg/ml of 2,4-dinitrophenylhydrazine.

The 4OHCP derivative was prepared as described under Experimental and analyzed by LC–MS in order to confirm the identity of the 4OHCP derivative. The molecular mass of the derivative is 456 and the m/z is 457. The LC–MS spectra also contains peaks at 457, 459 and 461 with a peak height relation of 100:65:10.6%, which is typical for the two chlorides in the 4OHCP 2,4-dinitrophenylhydrazone molecule. The NMR spectra also confirmed that the prepared derivative was 4OHCP 2,4-dinitrophenylhydrazone.

3.2. Chromatography

4OHCP as the 2,4-dinitrophenylhydrazone derivative was retained on a Spherisorb S3ODS2 (100 × 4.6 mm I.D.) column and a pre-column

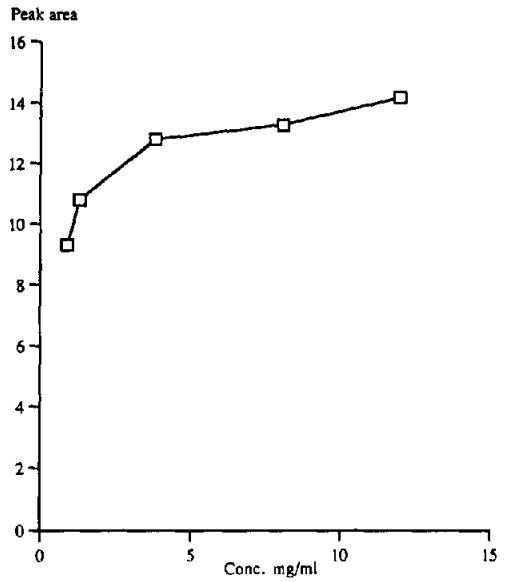

Fig. 2. Influence of the added concentration of 2,4-dinitrophenylhydrazine on the formation of the 4OHCP 2,4-dinitrophenylhydrazone derivative.

packed with similar material (10 × 3.2 mm I.D.) The mobile phase was a phosphate buffer pH 7 containing 40% (v/v) acetonitrile. The retention time was 9.6 min.

Typical chromatograms obtained from pooled blank plasma, plasma spiked with 4OHCP (22 ng/ml) and plasma from a patient who received CP intravenous, are shown in Fig. 3.

Additional peaks in the chromatogram are due to endogenous compounds (in the front, at 8.2 and at 11.4 min), excess of reagent (at 4.2 min) and impurities in the reagent (at 7.8 min). Late-eluting impurities from the reagent appeared after 20 and 40 min. In an attempt to avoid late-eluting impurities, 2,4-dinitrophenylhydrazine was recrystallized in butanol. This effort was unfortunately without success. However, the time between consecutive injections was optimized so that the 4OHCP peak was free from coeluting interfering compounds.

The injection volume was 40 μl. Injection of a larger sample volume did not increase the sensitivity as the column efficiency decreased dramatically. This is probably an effect of the high concentration of acetonitrile (70%) in the injected sample.

3.3. Stability

As discussed above 4OHCP is very unstable. It is therefore important to know the stability of 4OHCP during sample handling, storage, sample preparation and derivatization.

The stability of 4OHCP in human blood has been studied at 37 and 4°C. In Fig. 4 it is demonstrated that the half-life ($t_{1/2}$) of 4OHCP is ca. 4 min at 37°C, whereas no degradation was observed after 1 h at 4°C. This observation indicates that it is important to chill the blood samples from the patients in an ice bath immediately after collection.

At room temperature the plasma $t_{1/2}$ for 4OHCP is ca. 80 min, but if the plasma is deproteinized with acetonitrile, 4OHCP was stable for at least 1 h (Fig. 4).

The optimal pH for the derivatization of 4OHCP with 2,4-dinitrophenylhydrazine is 2. However, at pH 2 the concentration of 4OHCP was found to decrease 50% within 1 h. Therefore, it is important to add the reagent to the sample before or immediately after the sample has been acidified.

It was observed that 4OHCP is stable at −70°C for at least four months in plasma, plasma deproteinized with acetonitrile or as the 2,4-dinitrophenylhydrazone derivative.

3.4. Calibration curve, precision and recovery

A linear calibration curve ($R^2 = 0.996$) was obtained in the concentration range studied (22–2180 ng/ml). The reproducibility of the method is presented in Table 2. The relative standard deviation of the within-day precision is less than 13%, even at low plasma concentrations of 4OHCP (22 ng/ml). The relative recovery of 4OHCP is $\geq 97\%$ (see Table 2). The recovery was obtained by comparing the peak area obtained after derivatization of 4OHCP in plasma

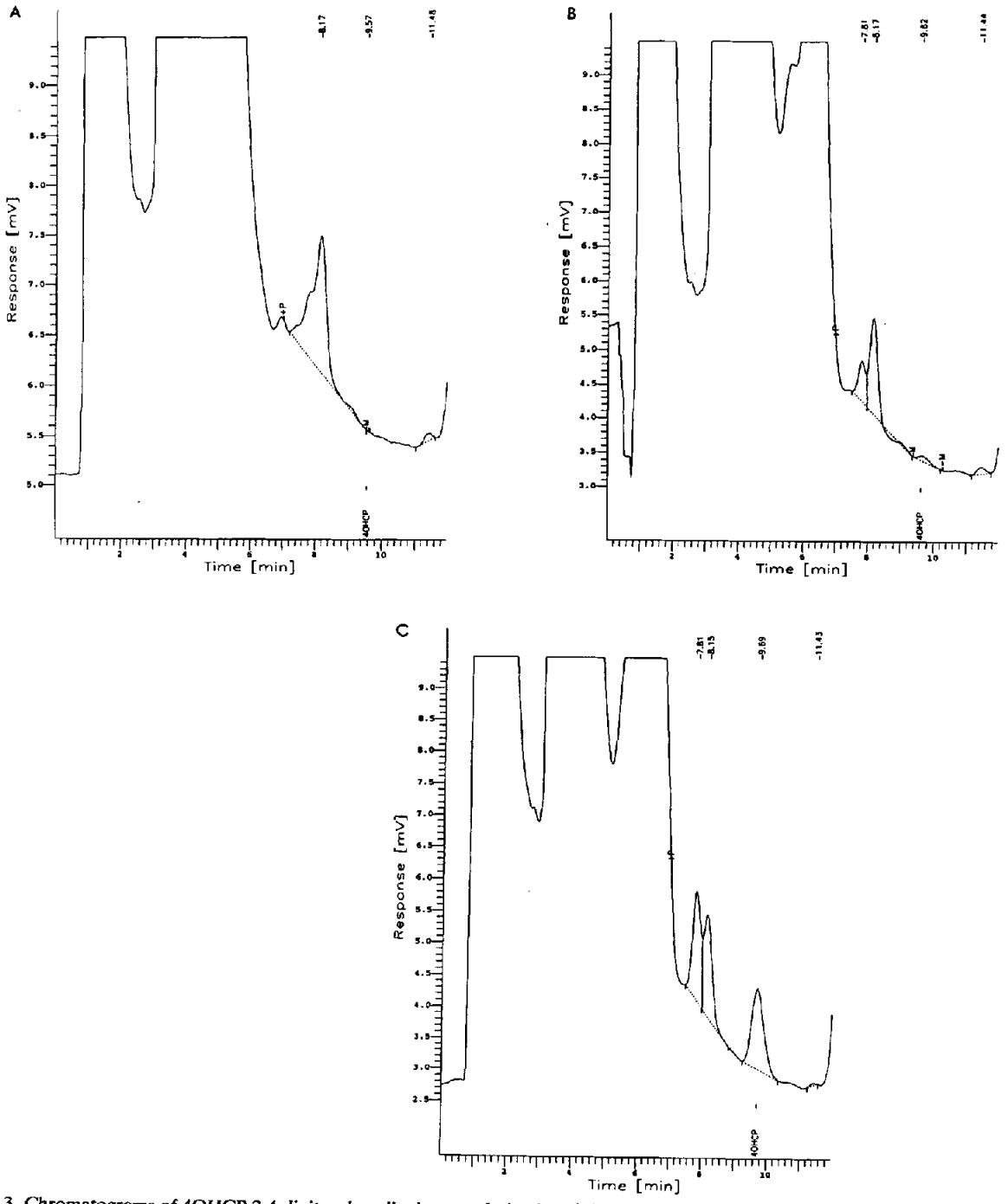

Fig. 3. Chromatograms of 4OHCP 2,4-dinitrophenylhydrazone derivative. (A) Blank plasma, (B) plasma spiked with 4OHCP (22 ng/ml) and (C) patient plasma 50 min after an intravenous bolus dose of CP (1100 mg). Chromatographic conditions: columns, Spherisorb S3ODS2 (10 × 3.2 mm I.D., 3 μm) and Spherisorb S3ODS2 (100 × 4.6 mm I.D., 3 μm). Mobile phase: phosphate buffer pH 7 ($\mu = 0.02$) containing 40% acetonitrile. Flow-rate: 1 ml/min. UV detection at 357 nm.

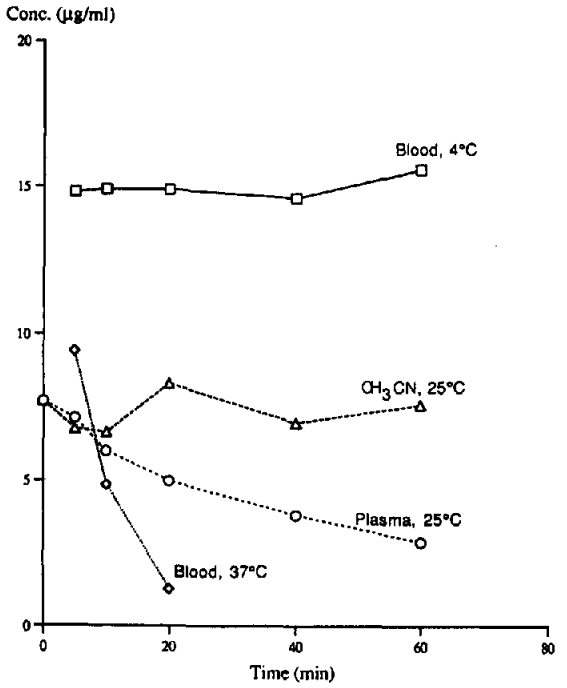

Fig. 4. Stability of 4OHCP in blood at 37°C and at 4°C , in plasma at 25°C, and in plasma deproteinized with acetonitrile at 25°C. Chromatographic conditions as in Fig. 3.

with the peak area obtained after derivatization of 4OHCP in water. It was not possible to determine the absolute recovery, since the concentration in the "batch-prepared" 4OHCP 2,4-dinitrophenylhydrazone derivative solution was too low. This may be due to a high water content in the derivative or unstability of the derivative in the absence of excess of reagent.

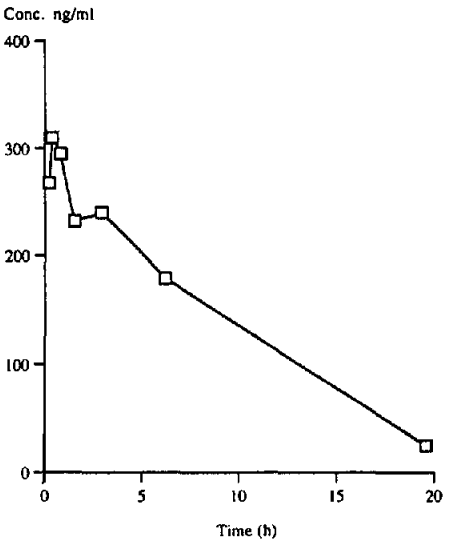

Fig. 5. Plasma concentration of 4OHCP in a patient with breast cancer, who had received an intravenous bolus dose of cyclophosphamide (1100 mg).

3.5. Concentration of 4OHCP in plasma

Fig. 5 demonstrates the plasma concentration of 4OHCP during 19 h in a patient with breast cancer who received an intravenous bolus dose of CP (1100 mg).

Studies of the pharmacokinetics and pharmacodynamics of 4OHCP are in progress.

3.6. Stereoselectivity

CP contains an asymmetrical phosphor atom and exists in two enantiomeric forms. It has been

Table 2
Precision and recovery of 4OHCP in plasma

Concentration (ng/ml)	n	Relative standard deviation (%)		Relative recovery (%)
		Within-day	Between-days	
1090	7	2.7		98
272	8	3.6		97
33	8	8.7		97
22	8	13		97
813	4		3.9	

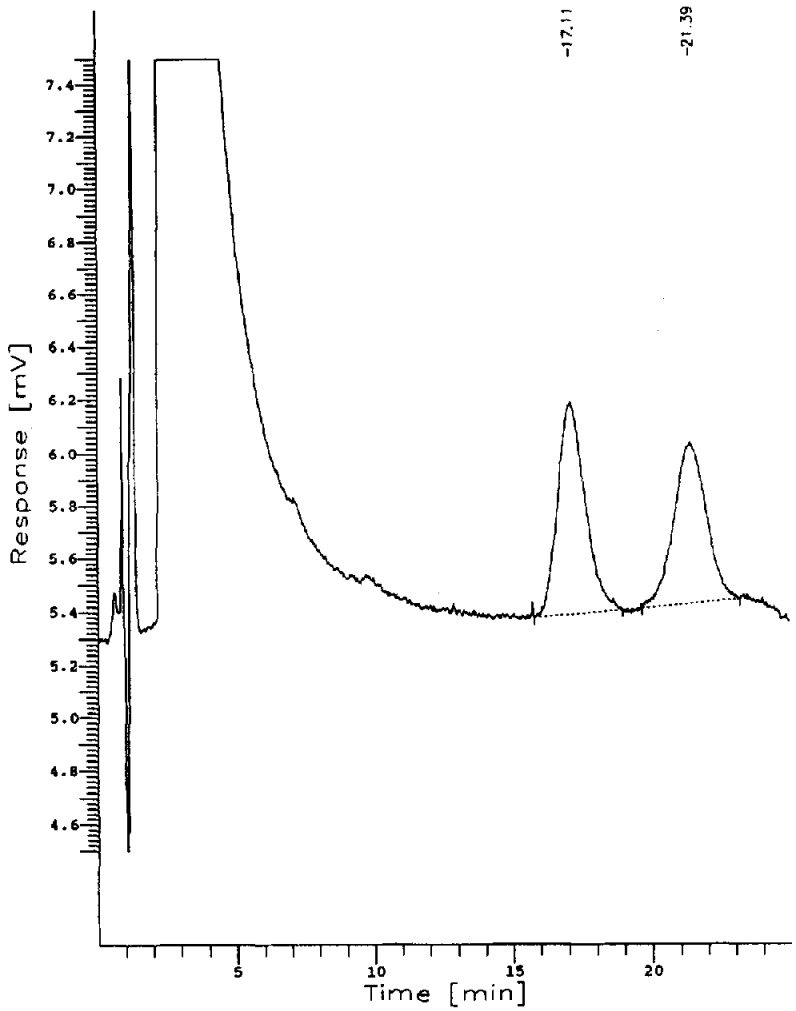

Fig. 6. Separation of *R*- and *S*-4OHCP as their 2,4-dinitrophenylhydrazone derivatives. Column: Chiral-AGP (100 × 4.0 mm I.D., 5 μm). Mobile phase: phosphate buffer pH 7.2 ($\mu = 0.02$) containing 5% acetonitrile. Flow-rate: 1.0 ml/min. UV detection at 357 nm. The elution order of the enantiomers was not determined.

observed that the *S*-(−)-enantiomer is twice as effective as the *R*-(+)-form in killing tumour cells [11].

In the clinic, CP is administered as a racemic mixture of the two forms, but there is little information about the disposition of the enantiomers in human. This is probably due to the lack of methods to separate and quantitate the enantiomers.

Recently, two chromatographic methods for the determination of *R*- and *S*-CP have been developed [12,13]. Holm et al. [14] used one of them [13] and found preponderance of *R*-CP in rabbits, indicating a more rapid metabolism of the *S*-enantiomer. In humans, the *R*/*S* ratio was 1.0 initial and 0.8 at 4 h after i.v. administration of CP. However, no method for the determination of *R*- and *S*-4OHCP has been reported.

In Fig. 6 it is demonstrated that the two enantiomers of 4OHCP as their 2,4-dinitrophenylhydrazone derivative can be separated on a Chiral-AGP column with a mobile phase of

phosphate buffer pH 7.2 containing 5% acetonitrile. However, in order to quantitate the enantiomers of 4OHCP in plasma after intravenous administration of CP, the method has to be further developed.

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