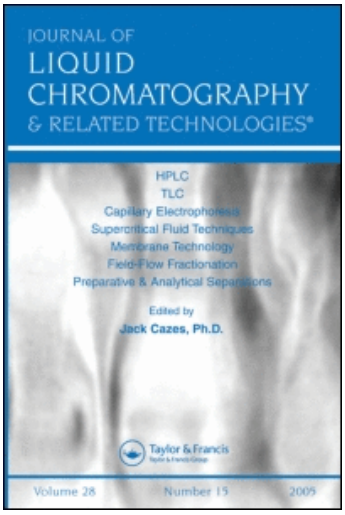

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 06 July 2000

To cite this Article Huitema, A. D. R. , Tibben, M. M. , Kerbusch, T. , Bosch, J. J. Kettenes-van den , Rodenhuis, S. and Beijnen, J. H.(2000) 'HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE STABILIZED CYCLOPHOSPHAMIDE METABOLITE 4-HYDROXYCYCLOPHOSPHAMIDE IN PLASMA AND RED BLOOD CELLS', Journal of Liquid Chromatography & Related Technologies, 23: 11, 1725 — 1744
To link to this Article: DOI: 10.1081/JLC-100100448
URL: http://dx.doi.org/10.1081/JLC-100100448

J. LIQ. CHROM. & REL. TECHNOL., 23(11), 1725–1744 (2000)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE STABILIZED CYCLOPHOSPHAMIDE METABOLITE 4-HYDROXYCYCLO-PHOSPHAMIDE IN PLASMA AND RED BLOOD CELLS

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ABSTRACT

A reversed-phase high performance liquid chromatographic (HPLC) method for the determination of the activated cyclophosphamide (CP) metabolite 4-hydroxycyclophosphamide (4-OHCP) in human plasma and red blood cells is described. 4-OHCP is very unstable in biological matrices. Therefore, it was treated, immediately after sampling, with semicarbazide to form a stable

semicarbazone derivative, which was identified with electron spray mass spectrometry. The derivative was analysed with HPLC with ultraviolet (UV) detection at 230 nm. Sample pretreatment consisted of a liquid-liquid extraction with ethyl acetate, the chromatography system was a 25 cm C8 column (particle size 5 μm) with a mobile phase of acetonitrile-0.025 M potassium phosphate buffer (15:85 v/v) pH 6.0. After assay optimisation, the method was validated and stability studies were carried out. The method proved linear in clinically relevant concentrations (50-5000 ng/mL). The lower limit of quantitation was 50 ng/mL. Accuracy and precision were below 7% over the full concentration range. A calibration curve in plasma can also be used for the quantification of 4-OHCP in red blood cells. The derivative was found to be stable for at least 11 months when stored at -70 °C. A pharmacokinetic pilot study in a patient treated with 1000 mg/m^2 CP in combination with thioTEPA and carboplatin demonstrated the applicability of the assay for the determination of 4-OHCP in plasma and red blood cells.

INTRODUCTION

Cyclophosphamide (CP, 2-[bis-(2-chloroethyl)amino]-tetrahydro-2H-1,3,2,-oxazaphosphorine-2-oxide, Endoxan®) is widely used in cancer chemotherapy, both in conventional and high-dose regimens.[1,2] CP and its analogue ifosfamide, themselves, are inactive and need to be activated by the cytochrome P450 mixed function oxidase enzymes to exert cytotoxicity. First, CP is hydroxylated to 4-hydroxycyclophosphamide (4-OHCP) which is in equilibrium with its tautomeric form aldophosphamide. 4-OHCP is unstable and rapidly decomposes into phosphoramide mustard, which is considered the ultimate alkylating metabolite, and acrolein, which is responsible for urotoxicity. Oxidation of CP, 4-OHCP and aldophosphamide leads to the formation of the inactive metabolites 2-dechloroethylcyclophosphamide, 4-ketocyclophosphamide and carboxyphosphamide, respectively (Figure 1). Because 4-OHCP and aldophosphamide cannot be distinguished with common assays, the name 4-OHCP is used here to denote both 4-OHCP and aldophosphamide.

In contrast to phosphoramide mustard, 4-OHCP is able to pass cellular membranes.[1] Systemic concentrations of 4-OHCP may therefore reflect the intracellular activation state of CP. A higher concentration of 4-OHCP within red blood cells compared to plasma has been described.[3,4] Red blood cells have been proposed to be a carrier of 4-OHCP to the tumour site. Therefore, it is considered to be important to determine 4-OHCP both in plasma and in red blood cells.

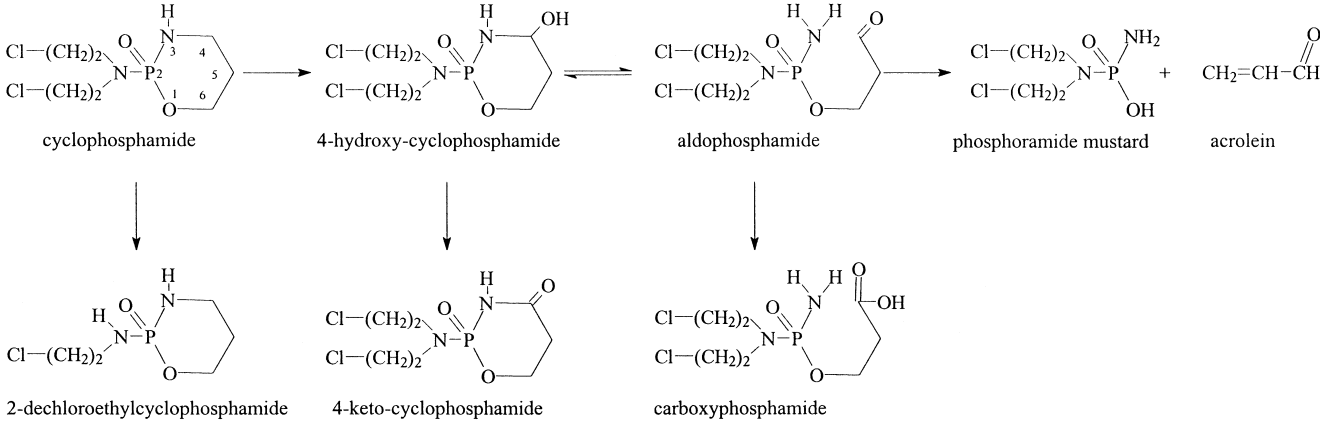

Figure 1. Metabolism of CP and its main metabolites; in horizontal direction the bioactivation of CP is depicted, vertical the inactivation processes.

Most methods for the determination of 4-OHCP are based on the liberation of acrolein from 4-OHCP, followed by trapping acrolein with 3-aminophenol. The fluorescent product 7-hydroxyquinoline is formed and is measured fluorimetrically[5,6] or with high performance liquid chromatography (HPLC) with fluorescence detection.[7] Using these methods 4-OHCP is determined indirectly. Although acrolein formed prior to sample collection will interfere with the analysis, interference is not likely to occur since acrolein reacts rapidly with plasma proteins. Because of the instability of 4-OHCP a rapid derivatization procedure to yield a stable product is required.

For gas chromatography coupled with mass spectrometry (GC-MS), 4-OHCP is derivatized with agents such as N,O-bis(trimethylsylil) trifluoroacetamide (BSTFA) and cyanohydrin[8] or O-(2,3,4,5,6-penta fluorobenzyl)hydroxylamine-HCl.[9] Not commercially available deuterium labelled internal standards have been used and extensive sample pre-treatment appeared necessary.

For the simultaneous determination of CP and some of its metabolites (including 4-OHCP), methods using thin layer chromatography after derivatization have been described.[10,11] These methods, however, lack the required sensitivity for the measurement of 4-OHCP in biological samples of patients treated with CP.

Various HPLC methods for the determination of 4-OHCP after direct derivatization have been published in the literature. Derivatization is carried out with 2,4-dinitrophenylhydrazine[12] or *p*-nitrophenylhydrazine.[13] Disadvantages of these methods are the chromatographic handling of excess reagent and late-eluting impurities.[12] Belfayol et al. described the determination of 4-OHCP in plasma after derivatization with semicarbazide (SCZ) followed by HPLC with UV detection at 230 nm[14] (see Figure 2 for the reaction scheme). SCZ has no absorption at this wavelength and thus excess reagent does not show up in the chromatogram. However, this method needs extensive sample pre-treatment to avoid interferences from endogenous compounds. No optimisation of the derivatization procedure and no determination of 4-OHCP with the method in red blood cells were described.

The aim of our study was to develop a simple method for the quantitation of 4-OHCP in plasma and red blood cells after direct derivatization with SCZ, suitable for pharmacokinetic studies in patients receiving CP. Furthermore, a detailed optimisation and stability study was carried out to validate each step in the handling of the samples, from collection on the ward up to analysis in the laboratory.

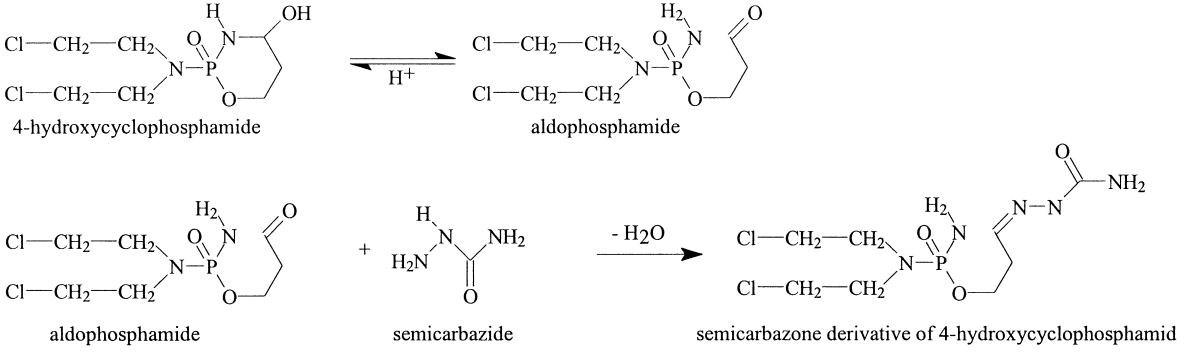

Figure 2. The derivatization of 4-OHCP with SCZ.

EXPERIMENTAL

Chemicals

4-Hydroperoxycyclophosphamide (4-OOHCP; purity > 95%) and all other CP metabolites were a kind gift of Dr. J. Pohl, Asta Medica (Frankfurt, Germany). Ethyl acetate and acetonitrile (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands), potassium dihydrogenphosphate (suprapure grade) was from Merck (Darmstadt, Germany). Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). A 2 M solution of SCZ in 50 mM potassium phosphate buffer (pH 7.4) was prepared. Blank plasma was obtained from the Central Laboratory of Blood Transfusion (Amsterdam, The Netherlands). Blank red blood cells were obtained from volunteers in the Slotervaart Hospital (Amsterdam, The Netherlands). Distilled water was used throughout and all other chemicals used were of analytical grade and used without further purification.

HPLC Equipment and Chromatography

The HPLC system consisted of a Thermo Separations Products Spectraseries (TSP, Fremont, CA, USA) model AS3000 automated injector with a 100 µL loop, a model P1000 solvent delivery system and a Spectra 100 variable wavelength UV detector (Spectra Physics, Santa Clara, CA, USA). Separation was carried out with a Prodigy 5 C8 column (250 x 4.6 mm, particle size 5 µm) (Phenomenex, Torrance, CA, USA), protected with a C8 guard column (Security Guard, Phenomenex). Retention times and peak areas were measured with a Datajet integrator which was part of a PC1000 data system (TSP).

The mobile phase consisted of acetonitrile-0.025 M potassium phosphate buffer pH 6.0 (15:85 v/v). The flow-rate was 1.0 mL/min, the column was operated at ambient temperature. The detection wavelength was 230 nm.

Sample Pre-Treatment

Immediately after collection in heparin-tubes, whole blood samples were placed on ice and centrifuged at 1,500 g for 3 min at 4°C. A 1.0 mL volume of plasma was added to a 5 mL polypropylene tube with 100 µL 2M SCZ. The remaining plasma was removed and the white blood cell layer was discarded with sufficient margins. Hereafter, 1 mL of the red blood cell layer was added to a 5 mL polypropylene tube with 100 µL 2M SCZ. Samples were whirlmixed for 30 s and stored at -70°C until analysis. After thawing, 100 µL of water and 2.0 mL of ethyl acetate were added. Samples were whirlmixed for 30 s and then

centrifuged for 10 min at 2,500 g. Exactly 1500 µL of the organic phase were transferred into a 2 mL eppendorf tube. The ethyl acetate layer was evaporated to dryness under a gentle stream of air at 30°C. A 40 µL volume of acetonitrile was added and the samples were whirlmixed for 30 s. After 1 h 125 µL of water were added and, again, the samples were whirlmixed for 30 s. Of this mixture, 50 µL were injected into the HPLC system.

Calibration

A stock solution of 4-OHCP was prepared by dissolving 4-OOHCP in water, which rapidly decomposes to hydrogen peroxide and 4-OHCP, resulting in a final concentration of 1.0 mg/mL 4-OHCP. This solution was further diluted in water in such a way that, after drug-free human plasma (1.0 mL) was spiked with 100 µL of the dilutions 4-OHCP concentrations of 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL in plasma were obtained. To these mixtures, 100 µL of the SCZ solution pH 7.4 were added and the samples were whirlmixed for 10 s and stored at -70°C until analysis. After thawing these calibration samples were processed as described for the blood samples.

Optimisation

The influence of the concentration of SCZ on the derivatization of 4-OHCP was investigated by adding 100 µL of various concentrations of SCZ (2, 1, 0.5, and 0.2 M) to 1.0 mL plasma samples with 250 or 2500 ng/mL 4-OHCP. After 10 min the samples were treated as described. The recovery obtained from the 2M SCZ solution was set at 100%; higher concentrations did not improve the yield of the analyte. Each sample was made in 5-fold.

The stability of 4-OHCP before derivatization was established by incubating 1.0 mL plasma spiked with 4-OHCP (final concentration of 2500 ng/mL) at ambient temperature for 1, 5, 10, 30, and 60 min before adding 100 µL 2 M SCZ, all in triplicate. The recovery of the sample immediately derivatized with SCZ was set at 100%.

The optimal conditions for the derivatization of 4-OHCP were investigated by adding 100 µL of SCZ to 1.0 mL plasma spiked with 4-OHCP (final concentration of 250 or 2500 ng/mL), followed by incubation at ambient temperature for 0, 2, 10, 60, min and 24 h and at 60°C for 1 h, all in 4-fold. Immediately after incubation, samples were extracted with ethyl acetate as described. The recovery of the samples incubated at ambient temperature for 10 min reached the highest value and was set at 100%.

Validation

A three-run validation of the determination of 4-OHCP in plasma on the following parameters was performed: accuracy, within-day, and between-day precision, linearity, selectivity, and specificity, extraction efficacy, limit of detection (LOD), lower limit of quantitation (LLQ), stability of 4-OHCP-SCZ in plasma and in processed samples. The determination of 4-OHCP in red blood cells was validated using a single-run validation using a calibration curve in red blood cells together with quality control samples in plasma to establish the suitability of quantitation from plasma samples for the determination of 4-OHCP in red blood cells. The extraction efficacy of 4-OHCP-SCZ from red blood cells was compared with that from plasma by dividing the slope of the calibration curve in red blood cells by the slope of the calibration curve in plasma.

Quality control samples in plasma were prepared at 4-OHCP concentrations of 50, 250, 1000, and 5000 ng/mL. Six replicates of each quality control level were analysed simultaneously with calibration curves in three consecutive runs. The accuracy was defined as the percentage of the mean deviation from the nominal concentration.

The within-day and between-day precision were calculated with a one-way analysis of variance (ANOVA) using the analytical run as the group variable. From the ANOVA analysis the day mean square (DayMS), error mean square (ErrMS), and grand mean (GM) were obtained. Within-day and between-day precision were defined using Eqs. (1) and (2), respectively, where n is the number of replicates.

$$\text{within-day precision} = \frac{100 \times \sqrt{\text{ErrMS}}}{\text{GM}} \quad (1)$$

$$\text{between-day precision} = \frac{100 \times \sqrt{\frac{(\text{DayMS} - \text{ErrMS})}{\text{n}}}}{\text{GM}} \quad (2)$$

The linearity of three calibration curves was tested with the F-test for lack of fit, using a weight factor of $1/(\text{conc.})^2$ in order to avoid biasing in favour of the high standards.[15] The deviation of the measured concentration from the theoretical concentration is calculated for each calibration concentration.

The LOD was determined using a signal-to-noise ratio of 3. The LLQ was defined as the lowest calibration concentration with an accuracy and precision below 20%. The upper limit of quantitation was arbitrarily defined as 5000 ng/mL.

The extraction efficacy was investigated by dissolving approximately 300 μg 4-OOHCP in 2 M SCZ yielding a stock solution with a final concentration of 500 μg/mL 4-OHCP (as derivative). This solution was diluted in water to final concentrations of 2500 and 25000 ng/mL and 100 of these solutions were added to 1.0 mL of plasma and 100 μL of 2 M SCZ resulting in a final concentration of 250 and 2500 ng/mL 4-OHCP-SCZ in plasma. These samples were processed as described. The stock solution was diluted in 25% acetonitrile in water to final concentrations of 1136 and 11360 ng/mL corresponding to the final concentrations in the processed samples assuming a recovery of 100%. Both the processed and the non-processed samples were prepared in 6-fold and analysed together. The recovery was calculated as the ratio of the peak area of the processed samples and the non-processed samples.

Possible interference from endogenous compounds was investigated by the analysis of six different blank plasma and red blood cell samples. CP and all known metabolites of CP other then 4-OHCP were investigated for interference with the analytical method: 4-ketocyclophosphamide, carboxyphosphamide, phosphoramide mustard, 2-dechloroethylcyclophosphamide and didechloroethylcyclophosphamide. Carboplatin, thioTEPA and its main metabolite TEPA, granisetron, acetaminophen, temazepam, dexamethasone, oxazepam, and sodium-2-mercaptoethane sulphonate (MESNA) were tested co-medication frequently given within high-dose chemotherapy. All compounds were tested in a final concentration of 20 μg/mL in plasma.

For the stability study of 4-OHCP plasma samples were spiked with 250 or 2500 ng/mL 4-OHCP and immediately derivatized with 2 M SCZ. Samples were stored at -70°C for 1 day, 5 days (including 3 freeze-thawing cycles), 5 days, 2 weeks and 1 month all in triplicate. After the storage period the samples were treated as described and stored as dry samples, until analysis. After the period of 1 month all samples were analysed together with freshly prepared samples of the same concentration. Furthermore, plasma samples were spiked with 500 or 5000 ng/mL 4-OHCP and derivatized with 2 M SCZ. Samples were stored, in 6-fold, for 11 months at -20°C and -70°C and analysed together with freshly prepared samples.

The stability of the processed samples in the autosampler at room temperature was investigated, by re-analysing calibration samples after 35 hours storage in the autosampler. The stability was calculated by dividing the slope of the calibration curve after 35 hours by the slope of the original calibration curve.

Preparation, Isolation, and Mass Spectrometric Identification of 4-OHCP-SCZ

Approximately 300 μg 4-OOHCP was dissolved in 2M SCZ to yield a final concentration of 200 μg/mL of 4-OHCP (as derivative). The solution was extracted twice with 2.0 mL ethyl acetate. The organic layers were combined and evaporated to dryness under a gentle stream of air at 30°C. The residue was dissolved in 50% acetonitrile in water and injected in an electron spray VG Platform II ion spray mass spectrometer (Micromass, Altrincham, UK) for identification.

Analysis of Patient Samples

For analysis, plasma and red blood cell samples were obtained from a patient treated with a high-dose chemotherapy regimen consisting of 1000 mg/m^2 CP by a 1-hour infusion (t=0, start of infusion) followed by 265 mg/m^2 carboplatin by a 1-hour infusion and 40 mg/m^2 thioTEPA in a 1/2-hour infusion. ThioTEPA was given each 12 hours for 4 days, CP and carboplatin were administered each day for 4 consecutive days. Blood samples were obtained during the first day before start of the infusions and at t=30, 60, 90, 120, 150, 180, 210, 285, 330, and 390 min, and the next day before the infusions. Blood was collected using a double-lumen central catheter.[16] First, 3 mL of blood were withdrawn and discarded, whereafter 5 mL of blood were collected and placed on ice. The samples were centrifuged immediately (on the clinical ward) at 1500 g and 4°C for 3 min and subsequently 1.0 mL of plasma and red blood cells were derivatized with 2 M SCZ and stored at -70°C until analysis. All samples were analysed within 2 weeks after collection.

Statistics

All statistical calculations were done with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated p-values were 0.05 or less.

RESULTS AND DISCUSSION

Chromatography and Detection

The aim of this study was to develop and validate a simple assay for the determination of 4-OHCP in plasma and red blood cells. Recently, a bioanalytical assay for the determination of 4-hydroxyifosfamide after stabilization with SCZ was described.[17] The described chromatographic system was, how-

ever, unsuitable for the determination of 4-OHCP and the system had to be changed substantially (another type of C8 column, other composition of mobile phase with different pH) enabling separation of 4-OHCP-SCZ from interfering endogenous compounds. Since, many possible critical steps were identified in the described method, each step in the analysis of 4-OHCP (from sampling on the clinical ward until analysis in the laboratory) was optimised and extensively validated including the identification of the SCZ derivative of 4-OHCP by mass spectrometry.

The chromatographic system was optimized by varying the acetonitrile percentage and the pH of the mobile phase. The mixture with 15% acetonitrile and pH 6.0 using the Prodigy C8 column provided the best separation between 4-OHCP-SCZ and interfering compounds combined with an acceptable retention time. Figure 3 shows typical chromatograms of patient samples obtained before and after an 1-hour CP infusion (1000 mg/m^2). Although some potential interferences are present, separation was sufficient, resulting in a lower limit of quantitation of 50 ng/mL. No peaks were observed eluting after 19 min, resulting in a convenient total run-time of 20 min.

The ifosfamide metabolite 4-hydroxyifosfamide (4-OHIF) can be derivatized with SCZ in the same way as CP and was, therefore, considered as internal standard.[17] This metabolite is, however, not readily available and, therefore, less suitable for an internal standard. Furthermore, the reproducibility of the assay was excellent, which made the use of an internal standard unnecessary.

Sample Pre-Treatment and Recovery

Belfayol et al.[14] described a labor-intensive two-step liquid-liquid extraction (LLE) procedure for the separation of 4-OHCP-SCZ from interfering compounds. Optimal recovery using LLE was obtained with ethyl acetate. However, to avoid an interfering peak in the chromatogram, these investigators used a mixture of chloroform and ethyl acetate (1:3) followed, after evaporation, by reconstitution of the sample in the mobile phase. The resulting mixture was, again, extracted with chloroform. With our chromatographic system, we found that a single-step LLE with ethyl acetate was adequate. Chloroform and ethyl acetate and mixtures of them were investigated for the extraction recovery. Ethyl acetate yielded the highest recovery. Extraction at pH 3 resulted in a very low recovery. Increasing the pH to 12 yielded equal recovery as that at neutral pH. However, at this high pH more interfering compounds were extracted and thus extraction at neutral pH was preferred.

Reproducibility of the extraction was increased by transferring exactly 1500 μL of the organic layer into an Eppendorf tube instead of completely

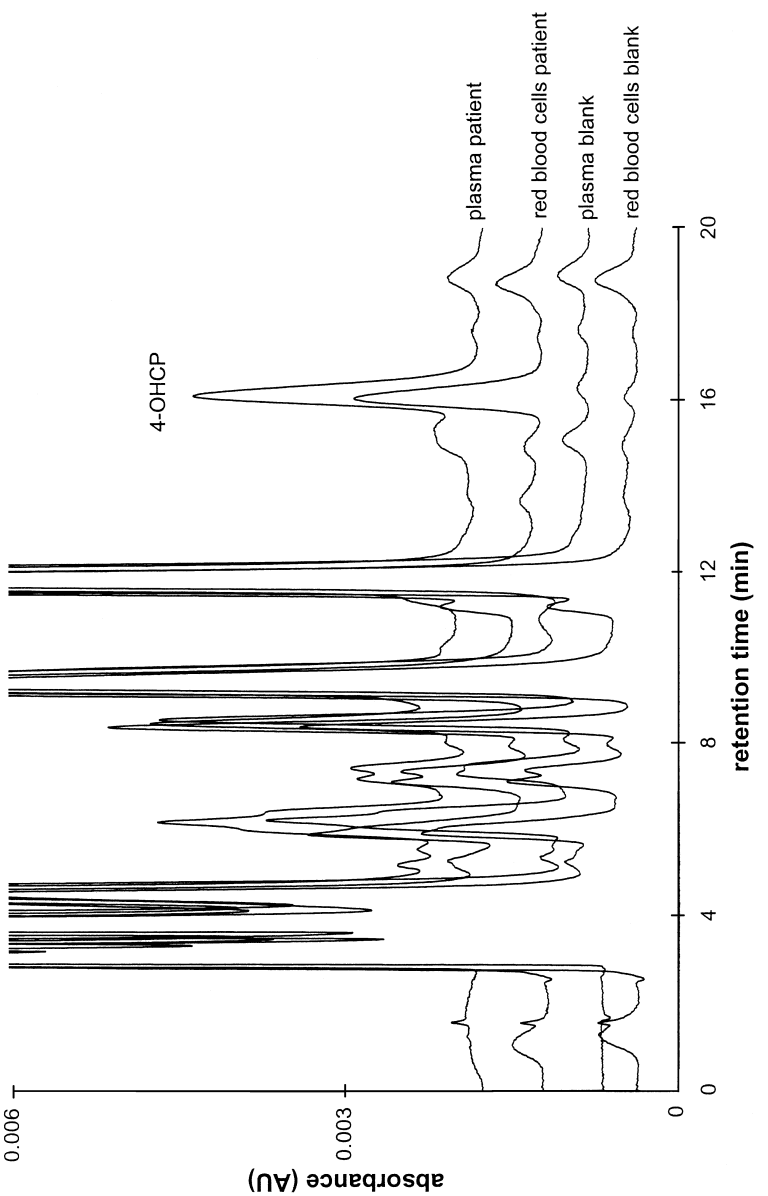

Figure 3. Representative chromatograms of plasma and red blood cell samples of a patient taken before and after a CP (1000 mg/m^2) infusion. Concentrations of 4-OHCP in plasma and red blood cells were 781 and 582 ng/mL, respectively.

transferring the ethyl acetate layer remaining after whirlmixing and centrifugation (approx. 1600 µL).

The extraction efficacy from plasma (± R.S.D.) was 34.6% (± 2.1) and 39.0% (± 2.0) for concentrations of 2500 and 250 ng/mL, respectively, with excellent reproducibility. The sample pre-treatment procedure with a single-step LLE with ethyl acetate was selected for further development of the assay. After addition of the 2M SCZ solution and subsequent storage at -70°C, the red blood cells were completely lysed. The extraction efficacy of 4-OHCP-SCZ from red blood cells was 91.4% compared to that from plasma.

Reconstitution of the dry sample in mobile phase did result in poor reproducibility. The dissolution rate of 4-OHCP-SCZ in the mobile phase proved to be low. Therefore, first, 40 µL of acetonitrile were added to the dry sample to dissolve 4-OHCP-SCZ. After 1 h 125 µL of water were added. This method resulted in complete and reproducible dissolution of the sample.

Optimisation

In Table 1 the influence of the SCZ concentration on the derivatization of 4-OHCP is shown. From the results it is clear that at least 0.5M SCZ is required for the derivatization of 4-OHCP.

The effects of time and temperature on the derivatization are shown in Table 2. Belfayol et al. used a derivatization with SCZ during 60 min at 60°C.[14] Our results show that under these conditions a considerable lower quantity of 4-

Table 1

Influence of the SCZ Concentration on the Derivatization of 4-OHCP at Ambient Temperature for Ten Minutes

	250 ng/mL		2500 ng/mL	
SCZ Conc. (M)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)
0.2	75.8	2.2	88.1	4.7
0.5	91.7	3.9	97.8	5.3
1	91.5	3.8	98.3	2.6
2	100	6.2	100	1.0

[a] R.S.D. = relative standard deviation.

Table 2

Effects of Time and Temperature on the Derivatization of 4-OHCPwith SCZ

Time (Min)	Temp. (°C)	250 ng/mL Recovery (%)	250 ng/mL R.S.D.* (%)	2500 ng/mL Recovery (%)	2500 ng/mL R.S.D.* (%)
0	20	88.7	14.7	48.1	25.8
2	20	96.7	8.9	68.1	4.2
10	20	100.0	5.3	100.0	6.4
60	20	91.8	2.7	95.6	7.0
1440	20	91.5	3.1	88.2	1.0
60	60	68.4	3.9	69.9	1.8

* R.S.D. = relative standard deviation.

OHCP-SCZ is formed than at 20°C. The degradation of 4-OHCP probably exceeds the formation of the stable derivative at the higher temperature. Therefore, a derivatization at room temperature for at least 10 min was chosen.

Figure 4 shows the stability of underivatized 4-OHCP in plasma. No loss of 4-OHCP occurred within 10 min. Therefore, clinical samples should be derivatized within 10 min after collection.

Validation

Data of the assay performance are presented in Table 3. Both accuracy and precision were below 7% which is far within the acceptable criteria.[18] For the three calibration curves in the validation, an F-test for lack of fit was performed. All p-values were higher than 0.05 indicating a linear relationship between concentration and peak area in the range tested. In Table 4 the deviation from the theoretical concentration and the relative standard deviation are given for all calibration concentrations. Correlation coefficients (r) of the calibration curves were higher than 0.998 as determined by least squares analysis.

The limit of detection was 40 ng/mL. The LLQ of the described method was 50 ng/mL. Both accuracy and precision at this concentration were below 7%.

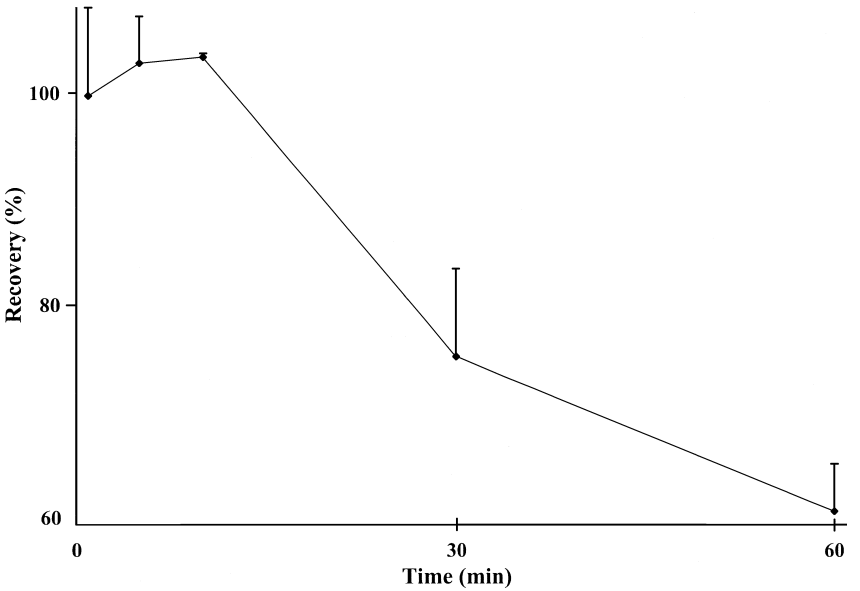

Figure 4. The stability of underivatized 4-OHCP in plasma at ambient temperature.

The calibration curve of 4-OHCP in red blood cells was linear in the same range as the calibration curve in plasma. The plasma quality control samples were interpolated on the calibration curve in red blood cells. The accuracy of all concentration levels was less than 13%. These data demonstrated that a calibration curve in plasma can be used to determine 4-OHCP in red blood cells.

Table 3

Accuracy and Precision of the Analysis of 4-OHCP in Plasma

Nominal Conc. (ng/mL)	Within-Day Precision(%)	Between-Day Precision(%)	Accuracy (%)	95% Confidence Interval
50	5.1	6.0	-4.1	-7.6 - -0.6
250	6.8	*	-0.6	-3.9 - 2.8
1000	4.2	3.5	2.8	0.1 - 5.3
5000	3.7	*	1.6	-0.3 - 3.4

* No statistically significant deviation between days of analysis was observed additional to the within-day precision.

Table 4

Calibration Curves: Deviations from the Nominal Concentration and Relative Standard Deviation at all Calibration Concentrations

Nominal Conc. (ng/mL)	Dev. (%)	R.S.D. (%)	n
50	-0.2	5.9	6
100	+2.0	2.9	6
250	-4.4	2.2	6
500	+0.7	3.7	6
1000	+0.5	4.8	6
2500	+0.9	3.2	6
5000	+0.6	1.6	6

Dev. = deviation from the nominal concentration; R.S.D. = relative standard deviation; n = number of replicates.

Analysis of 6 different blank plasma and red blood cell samples did not reveal any interfering endogenous compound jeopardizing the analytical outcome. The selected co-medication and metabolites did not interfere with the analysis. Furosemide, granisetron, oxazepam, and temazepam were detectable at 54, 31, 70, 110 min, respectively. These compounds did not interfere with the analysis using a run-time of 20 min. The CP metabolite 4-keto-cyclophosphamide was detectable (as SCZ derivative) at a retention time of 20.1 min. The LLQ of this metabolite in this assay was approximately 5 μg/mL which is unsuitable for the determination of this metabolite after treatment with CP.

In Table 5 the stability of 4-OHCP-SCZ during storage for various periods of time at -70°C in plasma is shown. The stability of 4-OHCP-SCZ in plasma during 11 months at -70°C and -20°C is shown in Table 6. After derivatization with SCZ samples are thus stable for at least 11 months stored at -70°C. Freeze-thawing of the samples did not influence the concentration of the sample. Moreover, it was shown that processed samples stored as dry sample at -70°C are stable for at least 1 month.

The recovery of 4-OHCP-SCZ in processed samples during storage for 35 hours in an autosampler was 105.5%, which indicates that after reconstitution samples are stable for a sufficient period of time to wait injection in an automated system.

Table 5

Stability of 4-OHCP-SCZ in Plasma After Storage at -70°C

Storage (Days)	250 ng/mL Recovery (%)	250 ng/mL R.S.D. (%)	2500 ng/mL Recovery (%)	2500 ng/mL R.S.D. (%)
1	103.2	2.3	105.6	4.9
5	103.6	2.3	105.7	6.1
5*	98.9	0.9	105.7	4.0
14	100.6	2.9	101.5	0.8
28	98.7	3.4	102.9	1.2

* Including 3 freeze-thawing cycles. R.S.D. = relative standard deviation.

Identification of the Derivatization Product

In Figure 5 the mass spectrum of the semicarbazide derivative of 4-OHCP is shown. The protonated molecular ion occurs at the expected m/z of 334.1 with characteristic chlorine isotopes at m/z 336.1 and 338.1. The clusters at m/z 356.1 and 372.1 originated from the sodium and potassium adduct of 4-OHCP-SCZ, respectively.

Analysis of Patient Samples

In Figure 6 the concentration vs. time curves of 4-OHCP in plasma and red blood cells of a patient treated with high-dose CP (in combination with

Table 6

Stability of 4-OHCP-SCZ in Human Plasma During Storage for 11 Months

Temp.	500 ng/mL Recovery (%)	500 ng/mL R.S.D. (%)	5000 ng/mL Recovery (%)	5000 ng/mL R.S.D. (%)
-20°C	65.0	2.4	77.6	4.1
-70°C	105.3	0.6	98.0	0.6

R.S.D. = relative standard deviation.

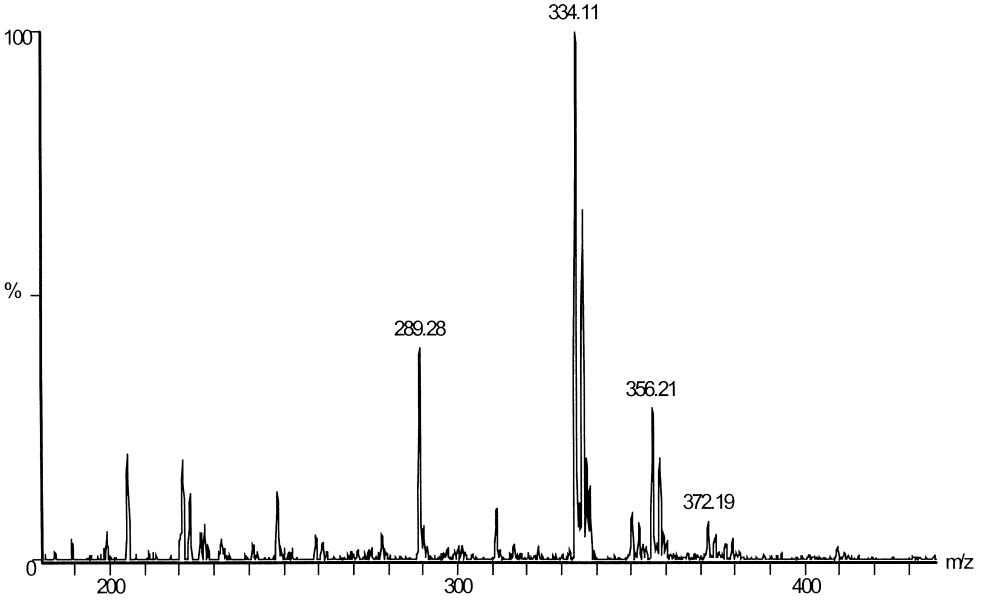

Figure 5. Mass spectrum of the semicarbazone derivative of 4-OHCP (MH^+, m/z 334.1).

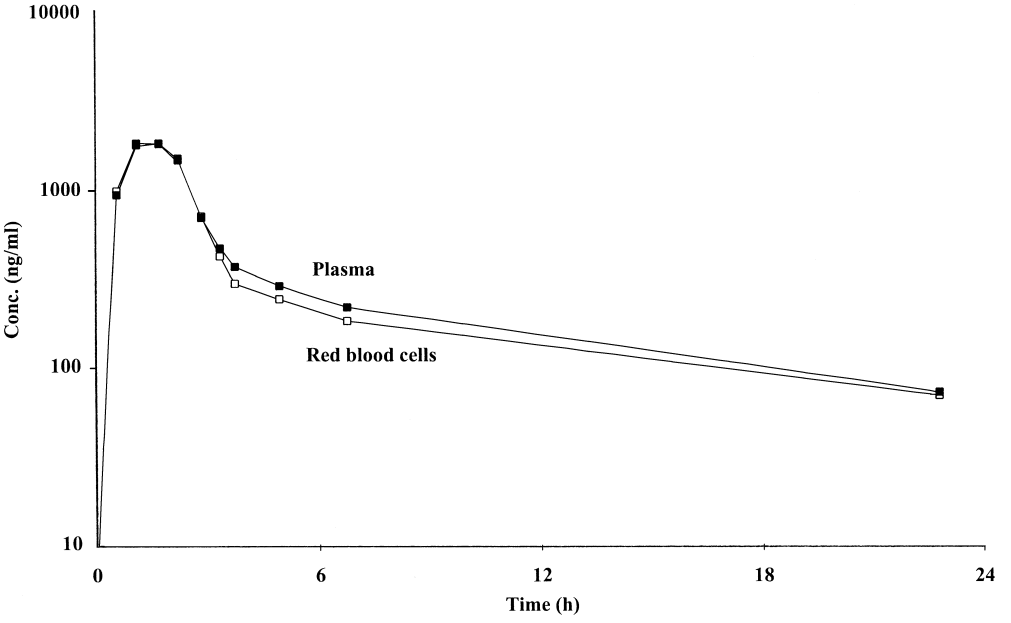

Figure 6. Concentration vs. time curves in plasma and red blood cells of a patient treated with a 1-hour infusion of CP (1000 mg/m^2) in combination with carboplatin (265 mg/m^2) and thioTEPA (40 mg/m^2).

thioTEPA and carboplatin) are shown. The concentration of 4-OHCP in plasma was equal to the concentration in red blood cells. The assay allowed the monitoring of 4-OHCP concentrations in plasma and red blood cells for at least 24 h after treatment with CP.

CONCLUSIONS

A simple and validated assay for the determination of 4-OHCP in plasma and red blood cells of patients treated with CP is described. The assay meets all the current requirements for bio-analytical assays. All steps in the sample handling, from the clinical ward to the analytical laboratory, have been validated. By derivatization with SCZ the unstable 4-OHCP is converted into 4-OHCP-SCZ which is stable for at least 11 months stored at -70°C.

ACKNOWLEDGMENTS

This research was supported with a grant from the Dutch Cancer Society (project NKI 97-1439). The authors would like to thank Dr. R. van Gijn and Dr. H. Rosing for their technical assistance and suggestions and Ms. J.M.A. Damen for running the mass spectra.

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Received September 20, 1999 Author's Revisions February 2, 2000
Accepted December 28, 1999 Manuscript 5177