ELSEVIER

Journal of Chromatography B, 745 (2000) 345–355

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simple and selective determination of the cyclophosphamide metabolite phosphoramide mustard in human plasma using high-performance liquid chromatography

Alwin D.R. Huitema[a,b,*], Matthijs M. Tibben[a], Thomas Kerbusch[a,b], J. Jantien Kettenes-van den Bosch[c], Sjoerd Rodenhuis[b], Jos H. Beijnen[a,b,d]

[a]*Department of Pharmacy and Pharmacology, The Netherlands Cancer Institute, Slotervaart Hospital, Louwesweg 6, 1066 EC, Amsterdam, The Netherlands*

[b]*Department of Medical Oncology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands*

[c]*Division of Biomolecular Mass Spectrometry, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3508 TG Utrecht, The Netherlands*

[d]*Division of Drug Toxicology, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3508 TG Utrecht, The Netherlands*

Received 11 January 2000; received in revised form 18 May 2000; accepted 18 May 2000

Abstract

A simple and selective assay for the determination of the alkylating cyclophosphamide metabolite phosphoramide mustard (PM) in plasma was developed and validated. PM was determined after derivatisation by high-performance liquid chromatography (HPLC) with ultraviolet detection at 276 nm. Sample pre-treatment consisted of derivatisation of PM with diethyldithiocarbamate (DDTC) at 70°C for 10 min, followed by extraction with acetonitrile in the presence of 0.7 *M* sodium chloride. Phase separation occurred due to the high salt content of the aqueous phase. The HPLC system consisted of a C_8 column with acetonitrile–0.025 *M* potassium phosphate buffer, pH 8.0, (32:68, v/v) as the mobile phase. The entire sample handling procedure, from collection at the clinical ward until analysis in the laboratory, was optimised and validated. Calibration curves were linear from 50 to 10 000 ng/ml. The lower limit of quantification and the limit of detection (using a signal-to-noise ratio of 3) were 50 and 40 ng/ml, respectively, using 500 μl of plasma. Within-day and between-day precisions were below 11% over the entire concentration range and the accuracies were between 100 and 106%. PM was found to be stable at −30°C for at least 10 weeks both in plasma and as a DDTC-derivative in a dry sample. A pharmacokinetic pilot study in two patients receiving 1000 mg/m^2 CP in a 1-h infusion demonstrated the applicability of the assay. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cyclophosphamide; Phosphoramide mustard

1. Introduction

Cyclophosphamide (CP) is one of the most frequently used anticancer agents, both in conventional-dose and in high-dose chemotherapy regimens [1].

*Corresponding author. Tel.: +31-20-512-4657; fax: +31-20-512-4753.

E-mail address: apahu@slz.nl (A.D.R. Huitema).

0378-4347/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00295-4

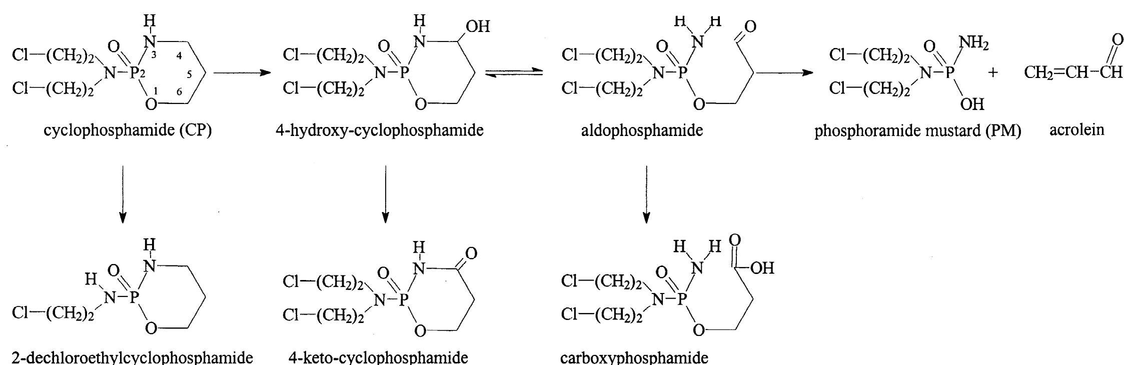

Fig. 1. Metabolism of cyclophosphamide.

CP is a prodrug with a complex metabolism consisting of both activation and inactivation routes. In Fig. 1 the metabolism of CP is shown. The first step in the activation of CP is 4-hydroxylation by cytochrome P450 enzymes. The resulting metabolite, 4-hydroxyCP, is in equilibrium with its ring-opened form, aldophosphamide. β-Elimination of acrolein from this unstable metabolite yields phosphoramide mustard (PM). PM is considered the ultimate alkylating metabolite of CP. PM is, however, not able to penetrate cell membranes and, therefore, only the intracellularly formed fraction is considered to be cytotoxic. In addition to the activation route, various metabolic deactivation routes exist for CP. Oxidation of CP, 4-hydroxyCP and aldophosphamide results in the formation of the inactive metabolites 2-dechloroethylCP, 4-ketoCP and carboxyphosphamide, respectively [2]. Metabolism of CP is complicated by the occurrence of auto-induction, which results in a reduced exposure to CP in time [1,2]. Since PM is the ultimate alkylating metabolite of CP and due to the very complex nature of CP metabolism, a simple method for the determination of PM in plasma of patients treated with CP would greatly facilitate detailed studies on the pharmacokinetics and metabolism of CP.

Few methods have been described for the determination of PM in plasma. Thin-layer chromatography has been used, but these methods lack the required sensitivity for pharmacokinetic studies [3–5]. Other methods have employed gas chromatography with flame ionisation detection [6] or mass spectrometric detection [7,8]. For the mass spectrometric measurements non-commercially available deuterated internal standards have been used [7,8]. For all methods extensive sample pre-treatment was necessary. Derivatisation has been performed with agents such as diazomethane [8], methylsilyltrifluoroacetamide [7] and boron trifluoride in methanol [6]. An ion-pair high-performance liquid chromatography (HPLC) method has been described with solid-phase extraction as sample pre-treatment [9]. Recently, a method for the determination of CP and 5 of its metabolites including PM has been published [10]. This method required extensive sample pre-treatment followed by separation and detection with LC–MS. The lower limit of quantification (LLQ) for PM of this method was 700 ng/ml (0.3 μM), which is, however, unsuitable for the determination of PM in plasma of patients treated with conventional-dose CP.

An HPLC method for ifosforamide mustard, the active metabolite of the CP isomer ifosfamide, has been described. Ifosforamide mustard was determined with UV detection after derivatisation with diethyldithiocarbamate (DDTC) and subsequent solid-phase extraction [11]. This method has not been applied for PM thusfar. However, also for this method sample pre-treatment is time-consuming and not each step of the assay has been validated. The LLQ of this method has been reported to be 100 ng/ml (0.45 μM).

The derivatisation reaction of PM with DDTC is shown in Fig. 2. Both a mono- and di-derivative of

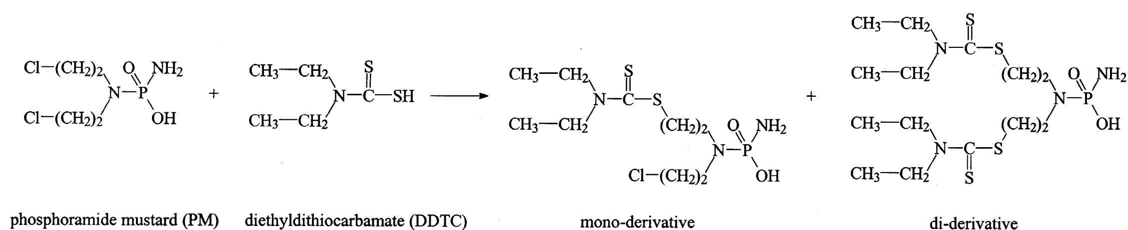

Fig. 2. Derivatisation reaction of phosphoramide mustard (PM) with diethyldithiocarbamate (DDTC).

PM can be formed since DDTC reacts with the chlorine in the two chloroethyl groups of PM. Kaijser et al. demonstrated that after optimisation of the derivatisation procedure only the di-derivative of ifosforamide mustard was formed [11].

The aim of the present study was to develop a more sensitive and, above all, simple assay for the determination of PM in plasma of patients treated with CP. DDTC was chosen as the derivatisation reagent and sample pre-treatment was considerably simplified. Moreover, HPLC–UV equipment present in hospital laboratories and commercially available reagents were used. To demonstrate the applicability of the method in pharmacokinetic studies, a detailed validation from collection at the clinical ward until analysis in the laboratory, including storage, was carried out.

2. Experimental

2.1. Chemicals

PM, CP and all other CP metabolites were a kind gift of Dr. Pohl (Asta Medica, Frankfurt am Main, Germany). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands), DDTC from Sigma (St Louis, MO, USA), hydrochloric acid, sodium hydroxide, dipotassium hydrogenphosphate and sodium chloride from Merck (Darmstadt, Germany) and semicarbazide hydrochloride from Acros (Geel, Belgium). Blank plasma was obtained from the Central Laboratory of Blood Transfusion (Amsterdam, The Netherlands). Distilled water was used throughout and all other chemicals used were of analytical grade and used without further purification.

2.2. Instrumentation

The HPLC system consisted of a Spectra Systems P1000 pump with a Spectra Series AS300 autosampler and a Spectra 100 UV detector (all from Thermo Separations Products, Fremont, CA, USA). Separation was carried out with a Prodigy C_8 column (250×4.6 mm, particle size 5 μm) (Phenomenex, Torrance, CA, USA), protected with a C_8 guard column (Security Guard, Phenomenex). Data were recorded and collected with the ChromQuest system (Thermo Separation Products). The mobile phase consisted of acetonitrile–0.025 *M* potassium phosphate buffer, pH 8.0 (32:68, v/v). The flow-rate was maintained at 1.0 ml/min and the system was operated at ambient temperature. The detection wavelength was 276 nm. On-line spectral analysis was carried out with a Waters 996 photo-diode array detection system (Waters, London, UK) connected to the HPLC system described above.

2.3. Sample pre-treatment

Whole blood samples were collected in heparin tubes and placed in an ice bath immediately followed by centrifugation at 4°C for 3 min at 1500 *g*. A 1.0-ml volume of plasma was transferred into a 1.5-ml Eppendorf tube containing 100 μl of a solution of 2 *M* semicarbazide HCl, 4 *M* sodium chloride and 0.1 *M* dipotassium hydrogenphosphate, pH 7.4 (2 *M* SCZ solution). After whirlmixing, the samples were stored at −70°C until analysis.

After thawing, 500 μl of the sample were trans-

ferred into a 2-ml Eppendorf tube, whereafter 50 μl of a freshly prepared solution of 10 mg/ml DDTC and 350 μl of a solution of 0.1 *M* dipotassium hydrogenphosphate and 1 *M* sodium chloride, pH 8.0 (PPB), were added. This mixture was incubated at 70°C for 10 min, followed by cooling of the samples in an ice bath. A 1.0-ml volume of acetonitrile was added and samples were whirlmixed for 30 s. After centrifugation (10 min, 10 500 *g* at 4°C), 600 μl of the acetonitrile layer were transferred into a 1.5-ml Eppendorf tube and were evaporated to dryness under a gentle stream of air at 40°C. The residue was reconstituted in 200 μl of mobile phase and whirlmixed for 30 s. The resulting solution was centrifuged for 5 min at 10 500 *g* and 50 μl of the clear supernatant were injected in the HPLC system.

2.4. Calibration

A stock solution of PM was prepared by dissolving an accurately weighed amount of PM in PPB resulting in a final concentration of 1.0 mg/ml. This solution was further diluted with PPB. Subsequently, 50 μl of the diluted solutions were added to 450 μl of plasma to which 50 μl of the 2 *M* SCZ solution were added corresponding to final concentrations of PM of 50, 100, 250, 500, 1000, 2500, 5000 and 10 000 ng/ml in plasma. Next, 300 μl of PPB were added (final volume of plasma phase=850 μl). Finally, 50 μl of a freshly prepared solution of 10 mg/ml DDTC in PPB were added. The sample pre-treatment of the calibration samples was performed as described in Section 2.3.

2.5. Optimisation of the derivatisation

Optimal incubation temperature and incubation duration were investigated at PM concentrations of 500 and 5000 ng/ml. Incubation was tested at ambient temperature, 40°C and 70°C for 0, 5, 10, 30 and 60 min, in duplicate.

The optimal DDTC concentration was determined at PM concentrations of 500 and 5000 ng/ml and DDTC concentrations of 0.1, 0.5, 1.0, 5.0 and 10 mg/ml, in duplicate.

2.6. Preparation, isolation and identification of PM derivative

A 500-μl volume of a solution of 1 mg/ml PM in PPB was derivatised with DDTC as described. After evaporation of the acetonitrile layer, the residue was dissolved in 200 μl of the mobile phase, followed by injection of the sample into the described HPLC system. Fractions of 1.5 ml of the mobile phase containing the PM derivative were collected and the derivative was extracted from the mobile phase by addition of 1 g of sodium chloride and 3.0 ml of acetonitrile. The acetonitrile layer was then transferred to a polypropylene tube and evaporated to dryness at 40°C under a gentle stream of air. The residue was reconstituted in acetonitrile followed by centrifugation (5 min, 10 500 *g*). The clear supernatant was again evaporated. The final residue was dissolved in 50% acetonitrile in water and injected into an electron spray VG Platform II mass spectrometer (Micromass, Altrincham, UK) for identification.

2.7. Validation

A three-run validation was carried on the following parameters: accuracy, within-day and between-day precision, linearity, selectivity and specificity, extraction efficacy, stability, limit of detection (LOD) and lower limit of quantification (LLQ).

2.8. Specificity and selectivity

Possible interference from endogenous compounds was investigated by the analysis of six different blank plasma samples. CP and all known metabolites of CP were investigated for interference with the analytical methods: 4-ketoCP, carboxyphosphamide, 4-hydroxyCP, 2-dechloroethylCP and didechloroethylCP. Interference from 4-hydroxyCP was investigated both with and without the addition of the 2 *M* SCZ solution. Carboplatin, granisetron, acetaminophen, temazepam, thioTEPA and its metabolite TEPA, topotecan, paclitaxel, ketoconazole, dexamethasone, oxazepam and sodium 2-mercaptoethane sulphonate (MESNA) are frequently co-administered in cyclophosphamide-based regimens

and were tested for interference. All compounds were tested at a final concentration of 20 μg/ml, which is equal to or exceeds each clinical relevant concentration. All interference studies were carried out with a run time of 120 min for the detection of possible late eluting peaks.

2.9. Accuracy, precision and linearity

Quality control samples were prepared at PM concentrations of 50, 100, 500, 2500 and 10,000 ng/ml. Five replicates of each level were analysed simultaneously with a calibration curve in three consecutive runs. The accuracy was defined as the percentage of the ratio of the observed concentration and the nominal concentration. Within-day and between-day precisions were calculated with a one-way analysis of variance (ANOVA) with 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 precisions are defined with Eqs. (1) and (2), respectively, where N is the number of replicates.

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

$$\text{Between-day precision (\%)} = 100 \times \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/N}}{\text{GM}} \quad (2)$$

The linearity of the three calibration curves was tested with the F-test for lack of fit [12] with a weight factor of $1/(\text{conc.})^2$ in order to avoid biasing in favour of the high standards. The deviation from the theoretical concentration was calculated for each calibration concentration.

2.10. Extraction efficacy

The PM-DDTC derivative was isolated as described and dissolved in PPB. The yield of the isolation step was unknown and therefore samples were diluted assuming a total recovery of 100%. The solution was further diluted in such a way that 100 μl of the final solutions, added to 500 μl of plasma with the 2 M SCZ solution, gave concentrations of 500 and 5000 ng/ml PM-DDTC. These samples were extracted with acetonitrile, as described, and 600 μl of the acetonitrile fraction were collected, evaporated to dryness and reconstituted in 200 μl of mobile phase. The stock solution of PM-DDTC in PPB was simultaneously diluted to concentrations corresponding to the final concentration of PM-DDTC in the reconstituted samples after pre-treatment assuming an extraction efficacy of 100%. All samples were prepared in duplicate and 50 μl of these solutions were injected into the HPLC system. The extraction efficacy was calculated as the ratio of the peak area in the processed and non-processed samples. In order to determine possible degradation of PM-DDTC during evaporation of the acetonitrile fraction, the same procedure was applied but without evaporation of the acetonitrile layer. The acetonitrile fraction was diluted with 0.025 M phosphate buffer pH 8.0 to obtain a concentration of 32% (v/v) of acetonitrile in buffer (equal to the composition of the mobile phase). The extraction efficacy was determined again from the ratio of the peak area of processed and non-processed samples.

2.11. Limit of quantification and limit of detection

The limit of detection (LOD) was determined using a signal-to-noise ratio of 3. The limit of quantification (LLQ) was defined as the lowest calibration concentration with an accuracy and precision below 20%. The upper limit of quantification was arbitrarily defined as 10 000 ng/ml.

2.12. Stability of PM

The stability of PM in plasma was investigated for samples kept at room temperature for 0, 0.5, 1, 3, 6 and 24 h; at −70°C for 1, 2, 4 and 10 weeks; at −30°C for 2 and 10 weeks and at −70°C with three freeze–thaw cycles. The stability of PM-DDTC in dry samples after evaporation of acetonitrile was investigated at −30 and −70°C for a period of 10 weeks. All conditions were tested at concentrations of 500 and 5000 ng/ml in triplicate. The samples were analysed together with a freshly prepared calibration curve.

Stability of the PM-DDTC derivative in the auto-sampler was investigated by re-analysing a calibration curve after 48 h. The stability was calculated by dividing the slope of the calibration curve after storage by the slope of the calibration curve analysed immediately after preparation.

2.13. Analysis of patient samples

Plasma samples obtained from two patients treated with CP (1000 mg/m^2/day) in a 1 h infusion followed by thioTEPA (80 mg/m^2/day) and carboplatin (265 mg/m^2/day) were analysed as described.

3. Results and discussion

3.1. Chromatography and detection

PM is non-volatile and shows no UV absorption. Therefore, PM requires derivatization for both gas chromatography and HPLC with UV detection. Derivatisation with DDTC resulted in a derivative with UV absorption at a relative high wavelength (276 nm). The pH of the mobile phase proved critical for the chromatographic behaviour and stability of PM-DDTC. Kaijser et al. have used ion-pair chromatography with cyclohexylamine at pH 7.0 for the separation of the DDTC derivative of ifosforamide mustard. Furthermore, they have reported that a pH below 6.5 resulted in decomposition of the derivative [11]. The robust Prodigy C_8 column tolerated a mobile phase with a pH up to 8.5. A pH of 8.0 resulted in good resolution and separation between the analyte and endogenous compounds without the need of an ion-pair forming agent. A pH of the mobile phase lower than 7.5 resulted in severe peak broadening. The optimal wavelength for the detection of PM-DDTC was 276 nm as determined with photo-diode array detection. At this wavelength, however, a relatively large peak due to excess reagent appeared in the chromatogram. Therefore, the acetonitrile percentage in the mobile phase was optimised for good separation between this peak and the analyte and an acceptable retention time. A mobile phase with 32% (v/v) acetonitrile provided optimal selectivity with an acceptable run time. Fig. 3 shows chromatograms of a calibration sample, a patient blank and a sample collected 4 h after a 1-h infusion of 1000 mg/m^2 CP. In the chromatogram, only one peak corresponding to PM-DDTC was observed at 11 min. This peak was isolated for structure confirmation. Parent CP also has two chloroethyl moieties and the DDTC derivative of CP was detected at a retention time of 33 min. The peak caused by excess DDTC appeared in the chromatogram without retention (2.3 min). The DDTC derivative of ifosforamide mustard showed a retention time of 10.5 min and was considered as internal standard. Ifosforamide mustard is, however, not commercially available and therefore less suitable as internal standard. Moreover, the assay showed accuracies and precisions amply fulfilling the requirements for method validation without the addition of an internal standard.

3.2. Sample pre-treatment

Since in biological samples 4-hydroxyCP readily decomposes into PM and acrolein, patient samples were placed on ice, immediately after collection. Plasma was separated at 4°C and subsequently treated with SCZ, which traps 4-hydroxyCP to the corresponding semicarbazone [11,13].

Sample pre-treatment for the determination of PM has usually been carried out by solid-phase extraction regardless of the derivatisation method [6–9,11]. Due to the polar nature of PM-DDTC, liquid–liquid extraction with non-polar solvents results in very low extraction recoveries. Due to the large final salt content of the sample after derivatisation (0.12 *M* SCZ and 0.7 *M* sodium chloride), addition of acetonitrile resulted in phase separation enabling liquid–liquid extraction with the more polar solvent acetonitrile. Moreover, the large salt content enhanced the stability of PM and ifosforamide mustard in plasma [9,11]. Reproducibility of the extraction procedure was increased by transferring an accurately measured aliquot of 600 μl of the acetonitrile fraction instead of the entire acetonitrile layer after sample pre-treatment. After reconstitution of the sample in the mobile phase, a final centrifugation step was carried out to avoid contamination of the HPLC system.

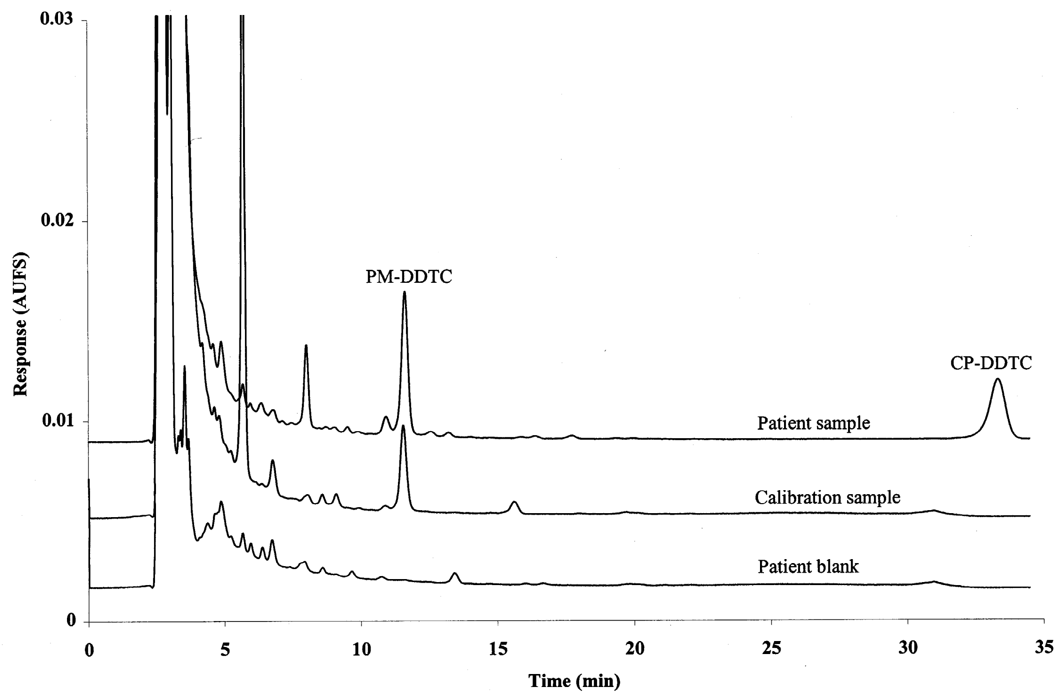

Fig. 3. Chromatograms of a calibration sample (500 ng/ml), a patients blank and a sample (981 ng/ml) collected 4 h after a 1-h infusion of 1000 mg/m^2 CP.

3.3. Optimisation of the derivatisation

Fig. 4 shows the formation of the DDTC derivative as a function of time during incubation at ambient temperature, 40°C and 70°C at a PM concentration of 5000 ng/ml. A similar result was obtained for a PM concentration of 500 ng/ml. Since, a maximum in derivatisation yield was

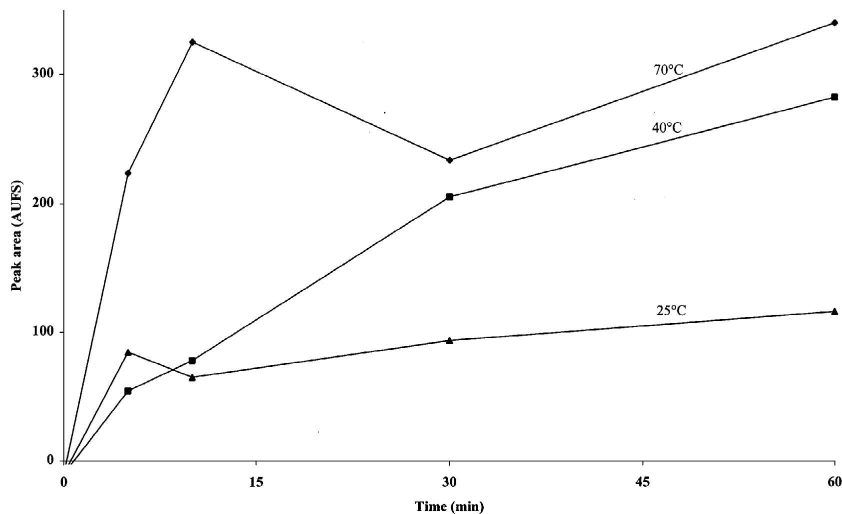

Fig. 4. Derivatisation yield vs. time during incubation at 70°C, 40°C and ambient temperature (PM concentration of 5000 ng/ml).

reached after 10 min of incubation at 70°C, these conditions were chosen for further development of the method.

In Fig. 5 the relation between the DDTC concentration and the derivatisation yield at a PM concentration of 5000 ng/ml is presented. Again, a similar result was obtained at a PM concentration of 500 ng/ml. The concentration of DDTC had a large impact on the chromatographic behaviour of the system due to a large tailing peak in the chromatogram caused by excess reagent. Therefore, a higher concentration of DDTC was not appropriate. Separation was similar and sufficient with both 5 and 10 mg/ml DDTC. Although a maximum yield was obtained at 5 mg/ml, a concentration of 10 mg/ml was chosen in order to reduce possible variability of the reaction. This DDTC concentration was much lower than that used by Kaijser et al. (100 mg/ml) [11].

3.4. Identification of the PM-DDTC derivative

The PM-DDTC peak with a retention time of 11 min, was isolated and identified using mass spectrometry. The peak of the protonated di-derivative of PM occurred at the expected m/z ratio of 447.2, with the sodium adduct at m/z 469.2. Furthermore, the dimer of PM-DDTC and the corresponding sodium adduct were observed in the mass spectrum.

3.5. Specificity and selectivity

Analysis of blank plasma of six different individuals did not show any interference. Analysis of a plasma sample with 20 μg/ml 4-hydroxyCP without the addition of SCZ did result in a large peak in the chromatogram at the same retention time as PM-DDTC due to the conversion of 4-hydroxyCP to PM. This peak was, however, not present in the chromatogram of the 4-hydroxyCP sample treated with SCZ. Nevertheless, another peak was seen at 12.5 min which did not interfere. Apparently, SCZ effectively blocks the conversion of 4-hydroxyCP to PM.

None of the other metabolites and co-administered drugs tested interfered with the analysis although a number were detected in the chromatogram. CP and some of its metabolites were detected (as DDTC derivatives) in the chromatogram. However, our assay did not allow quantification of these compounds with acceptable accuracy and precision. Moreover, the LLQ of most of these compounds was not adequate for the determination in patient samples. Since CP (as DDTC derivative) had a retention time of 33.0 min, the total run time was set at 35 min. ThioTEPA and TEPA gave late-eluting peaks at a test concentration of 20 μg/ml, which far exceeds normal therapeutic concentrations. No interference from these compounds was observed in patient samples, when a run-time of 35 min was used.

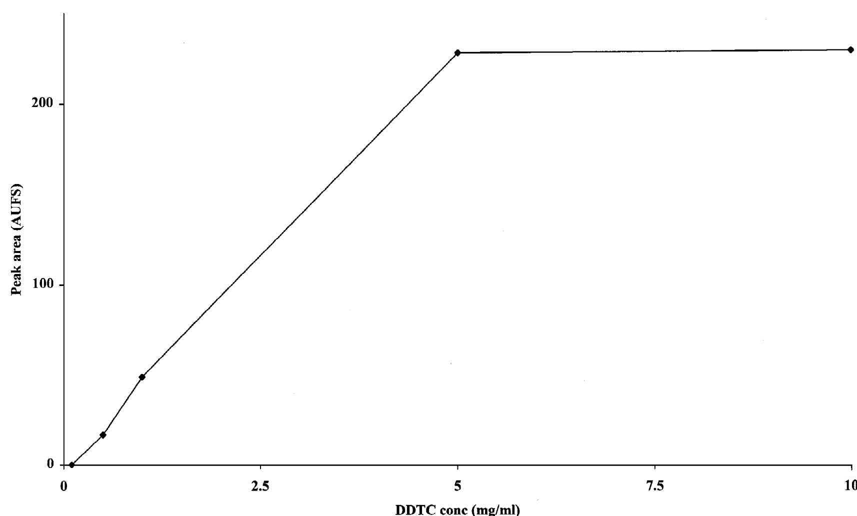

Fig. 5. Derivatisation yield vs. DDTC concentration during 10 min incubation at 70°C (PM concentration of 5000 ng/ml).

Table 1
Calibration curves: deviation from the theoretical concentration and relative standard deviation at all calibration concentrations[a]

Nominal PM conc. (ng/ml)	Dev. (%)	RSD (%)	N
50	0.0	6.2	6
100	+0.7	7.5	6
250	−1.7	5.4	6
500	0.0	5.1	6
1000	−0.2	3.6	6
2500	+0.5	5.2	6
5000	+0.3	4.7	6
10 000	+0.5	5.0	6

[a] Dev. = Deviation from the theoretical concentration; RSD = relative standard deviation; N = number of replicates at each level.

3.6. Accuracy, precision and linearity

All calibration curves proved to be linear over the concentration range of 50–10 000 ng/ml as confirmed with the F-test for lack of fit. Table 1 shows the mean deviation from the nominal concentration and the relative standard deviation at each calibration level for the three validation runs. The correlation coefficient (r) of each calibration curve was >0.997. Table 2 shows the accuracies and precisions of the assay. Although at the highest levels the accuracy was significantly higher than 100%, the accuracy was <106% at all levels. Within-day and between-day precisions at each quality control level were always below 11%.

3.7. Extraction efficacy

The recovery of the extraction procedure and the evaporation of the acetonitrile phase was 61.7±2.1% and 59.1±1.5% for 500 and 5000 ng/ml PM-DDTC in plasma, respectively. The extraction efficacy (without evaporation) was 65.6±2.4% and 65.4±0.8% for 500 and 5000 ng/ml PM-DDTC in plasma, respectively. The reproducibility of the extraction was excellent. Obviously, some degradation of PM-DDTC occurred during evaporation of acetonitrile. Since both the derivatisation and extraction procedure proved critical steps, it is recommended that all samples are treated simultaneously and in the same way as calibration curve and quality control samples.

3.8. LLQ and LOD

The LOD was determined to be 40 ng/ml at a signal-to-noise ratio of 3. The LLQ of this method was 50 ng/ml, which is comparable to the GC–MS methods (20–50 ng/ml) [6,8] but far better than the ion-pair HPLC or LC-MS methods (340–700 ng/ml) [9,10].

3.9. Stability

The stability of PM in plasma at room temperature is shown in Fig. 6. Since, for 1 h no degradation of PM was observed, samples should be derivatised within 1 h after thawing. The stability of PM in plasma at various conditions is shown in Table 3. PM in plasma and as PM-DDTC was stable at −70 and −30°C for at least 10 weeks. Hardy et al. demonstrated that at −20°C PM was stable in plasma for 11 days [9]. The recovery of the PM derivative after 48 h in the autosampler at ambient temperature was 99.1%.

Table 2
Accuracy and precision of the PM determination

Nominal conc. (ng/ml)	Within-day precision (%)	Between-day precision (%)	Accuracy (%)	95% C.I. of accuracy[a]
50	10.4	–[b]	103.4	97.7–109.1
100	5.1	–	100.2	97.6–102.9
500	6.2	2.4	104.0	100.2–107.8
2500	4.8	1.0	105.5	102.7–108.3
10 000	2.8	3.2	104.0	101.8–106.3

[a] 95% C.I. = 95% confidence interval.
[b] No statistically significant deviation between days of analysis was observed additional to the within-day precision.

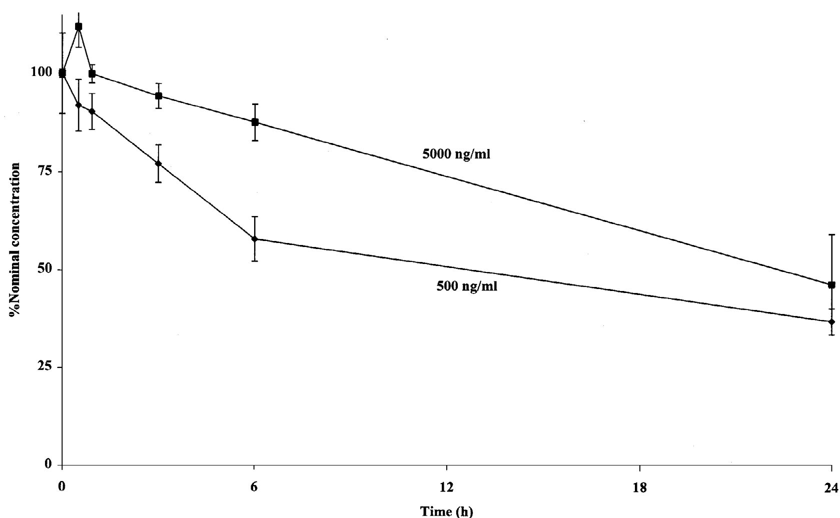

Fig. 6. Stability of PM in plasma during incubation at room temperature.

3.10. Patient samples

Fig. 7 shows the concentration–time curves of two patients treated with CP in a dose of 1000 mg/m^2. The patient with the highest PM concentration developed a reversible hemorraghic cystitis after this high-dose combination of CP, thioTEPA and carboplatin. CP-induced hemorraghic cystitis is believed to be caused by acrolein, a co-product of the formation of PM. These results demonstrated that the described method can be used for the determination of PM in the plasma of patients treated with CP.

4. Conclusion

In conclusion, a sensitive and simple method for the determination of PM in plasma of patients treated with CP is described. The assay meets the current requirements of the validation of a bioanalytical

Table 3
Stability of PM at −70 and −30°C

Condition	Time (days)	500 ng/ml		5000 ng/ml	
		Recovery (%)	RSD[a] (%)	Recovery (%)	RSD (%)
−70°C Plasma	7	107.0	3.8	101.0	4.6
−70°C Plasma	13	106.0	3.3	116.0	4.9
−70°C Plasma	28	109.0	1.4	108.0	3.1
−70°C Plasma	70	115.0	4.6	105.0	4.6
−70°C Plasma (3 freeze−thaw cycles)	7	103.0	1.7	99.2	5.0
−30°C Plasma	13	97.0	5.4	116.0	5.2
−30°C Plasma	70	112.0	4.1	108.0	2.6
−70°C dry sample (as derivative)	70	103.0	1.9	101.0	3.8
−30°C dry sample (as derivative)	70	99.7	4.0	96.2	5.7

[a] RSD = relative standard deviation.

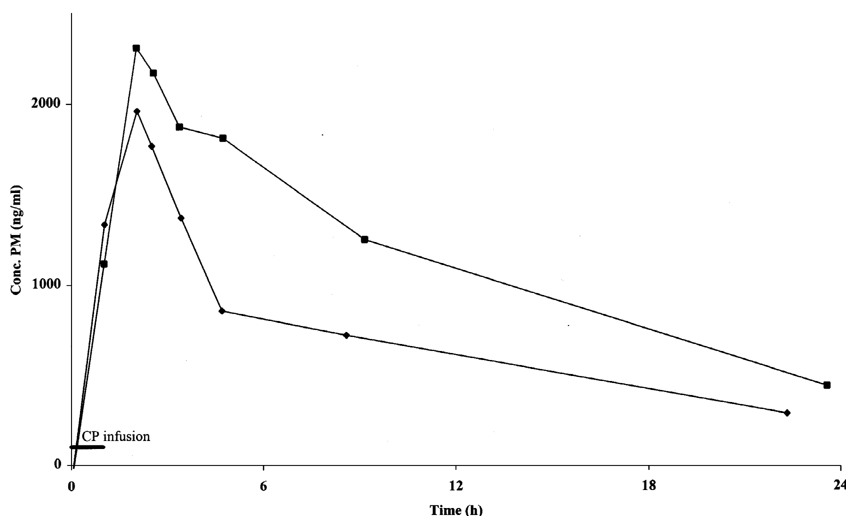

Fig. 7. Concentration–time curves of PM of two patients treated with CP in a dose of 1000 mg/m^2 in a 1-h infusion.

method and can therefore be used for pharmacokinetic studies.

Acknowledgements

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.

References

[1] E. van der Wall, J.H. Beijnen, S. Rodenhuis, Cancer Treat. Rev. 21 (1995) 105.
[2] M.J. Moore, Clin. Pharmacokinet. 20 (1991) 194.
[3] A.F.A. Hadidi, J.R. Idle, J. Chromatogr. 427 (1988) 121.
[4] J.F. Powers, N.E. Sladek, Cancer Res. 43 (1983) 1101.
[5] R.F. Struck, D.S. Alberts, Cancer Treat. Rep. 68 (1984) 765.
[6] F.D. Juma, H.J. Rogers, J.R. Trounce, Br. J. Clin. Pharmacol. 10 (1980) 327.
[7] A.A. Srigritsanapol, K.K. Chan, J. Pharm. Biomed. Anal. 8 (1994) 961.
[8] I. Jardine, C. Fenselau, M. Appler, M.-N. Kan, R.B. Brundrett, M. Colvin, Cancer Res. 38 (1978) 408.
[9] R.W. Hardy, M.J. Moore, C. Erlichman, S.J. Soldin, Ther. Drug Monit. 9 (1987) 221.
[10] T.F. Kalhorn, S. Ren, W.N. Howald, R.F. Lawrence, J.T. Slattery, J. Chromatogr. B 732 (1999) 287.
[11] G.P. Kaijser, J.H. Beijnen, E. Rozendom, A. Bult, W.J.M. Underberg, J. Chromatogr. B 686 (1996) 249.
[12] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Micotte, L. Kaufman, in: Chemometrics: A Textbook, Elsevier, New York, 1988, p. 84.
[13] L. Belfayol, L. Guillevin, K. Louchahi, O. Lortholary, A.M. Bosio, F. Fauvelle, J. Chromatogr. B 663 (1995) 395.