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# Sensitive gas chromatographic determination of the cyclophosphamide metabolite 2-dechloroethylcyclophosphamide in human plasma

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**Abstract**

Cyclophosphamide (CP) is one of the most frequently used anticancer agents. It is a prodrug requiring activation before exerting cytotoxicity. CP is deactivated to 2-dechloroethylcyclophosphamide (2-DCECP) with formation of an equimolar amount of chloroacetaldehyde. The aim of this study was to develop and validate a sensitive and simple assay for 2-DCECP in plasma of patients treated with CP. Sample pre-treatment consisted of solid-phase extraction of 500 μl of plasma over OASIS HLB (1 ml) cartridges with trofosfamide as internal standard. Separation and detection of underivatized 2-DCECP was performed with capillary gas chromatography with nitrogen/phosphorous selective detection. Extraction recovery of 2-DCECP exceeded 87%. No interference from endogenous compounds, other metabolites of CP and frequently co-administered drugs was detected. The assay was linear in the range of 5–5000 ng/ml in plasma. Accuracy, within-day and between-day precision were less than 11% for the complete concentration range. In plasma, 2-DCECP was stable for at least 1 month when kept at −70°C. Analysis of samples from patients treated with CP demonstrated the applicability of the assay. In conclusion, a sensitive and simple assay for 2-DCECP in plasma, which meets the current requirements for bioanalytical assays, was developed. 

*Keywords:* Cyclophosphamide; 2-Dechloroethylcyclophosphamide

## 1. Introduction

Cyclophosphamide (CP) is one of the most frequently used antineoplastic agents in conventional and high-dose regimens for both hematological malignancies and solid tumors [1,2]. CP is a prodrug with a complex metabolism (Fig. 1). The drug is activated by the cytochrome P450 to 4-hydroxycyclophosphamide/aldophosphamide. β-Elimination of acrolein from 4-hydroxycylophosphamide results in the formation of the ultimate alkylating metabolite, phosphoramide mustard. A second metabolic

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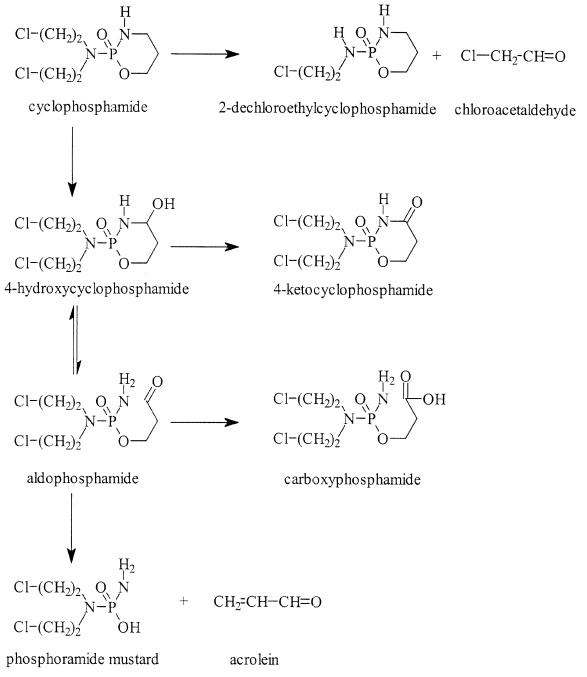


Fig. 1. Metabolic scheme of cyclophosphamide.

route of CP is side-chain oxidation to yield 2-dechloroethylcyclophosphamide (2-DCECP). Chloroacetaldehyde, formed in equimolar amounts with the dechloroethylation of CP, is suspected to be neurotoxic, nephrotoxic and cytotoxic [3]. Ifosfamide is a structural isomer of CP and shows a similar metabolic scheme. Side-chain oxidation of ifosfamide, however, results in the formation of two dechloroethylated metabolites, 2-dechloroethylifosfamide and 3-dechloroethylifosfamide of which the latter metabolite is similar to 2-DCECP.

For the last decade, CP has frequently been used in various high-dose combinations with hematological support. The very high doses employed in these regimens may cause shifts in the metabolic profile [2,4]. The use of combinations of drugs may lead to drug–drug interactions which may also have a major impact on metabolism [2,5]. Furthermore, CP shows autoinduction which results in an increased clearance of CP and an increased formation of its metabolites over time [2]. Few data on the plasma pharmacokinetics of 2-DCECP, however, exist although it may provide pivotal insight into the metabolism, autoinduction and thus therapeutic efficacy/toxicity of CP [2,6].

Various assays for 2-DCECP (or 3-dechloroethylifosfamide) have been described [7–13]. 2-DCECP has been determined underivatized using gas chromatography with nitrogen/phosphorous selective detection after liquid–liquid extraction with ethyl acetate or chloroform. The recovery of these extraction procedures was, however, below 40% [9,12]. In order to increase sensitivity, solid-phase extraction (SPE) over $C_8$ or $C_{18}$ columns with a recovery of 70–90% has also been used [7,10,13]. Detection sensitivity has been increased by derivatization of 2-DCECP with agents like trifluoroacetic acid anhydride (TFAA) [7,8] or *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide [11] followed by gas chromatography with mass spectrometric detection. Baumann et al. analyzed 2-DCECP underivatized in human plasma with liquid chromatography coupled with mass spectrometric detection [13]. One major drawback of the described methods is, however, that they have been developed primarily for the parent compound and not for the metabolite. The analytical conditions, including sample pre-treatment, separation and detection, have therefore not been optimized for the determination of the metabolite. Moreover, most of these methods have been developed for the determination of series of metabolites, which resulted in very labour-intensive methods with impaired sensitivity.

Therefore, the aim of this study was to develop a sensitive and simple assay for the determination of 2-DCECP in plasma of patients treated with CP, which can support ongoing studies in our Institute into the pharmacokinetics and pharmacodynamics of high-dose chemotherapy with CP.

## 2. Experimental

### 2.1. Chemicals

2-DCECP, CP and all other metabolites were a kind gift of Dr. Pohl (Asta Medica, Frankfurt am Main, Germany). ThioTEPA (*N*,*N*′,*N*″-triethylenethiophosphoramide, Ledertepa) was obtained from AHP Pharma (Hoofddorp, The Netherlands). TEPA (*N*,*N*′,*N*″-triethylenephosphoramide, purity>98%)

was synthesized as described in detail previously [14]. All gases used were obtained from Hoekloos (Schiedam, The Netherlands), methanol and ethyl acetate were from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid anhydride (TFAA) from Acros (Geel, Belgium) and diphenylamine from Merck (Darmstadt, Germany). 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. Various cartridges for SPE were used: OASIS HLB 1 ml (Waters, Milford, MA, USA), $C_{18}$, $C_2$, silica, phenyl and cyano stationary phases (all Bond Elut,Varian, Middelburg, The Netherlands).

### 2.2. Instrumentation

Measurements were performed with a gas chromatograph (HP 5890 series II, Hewlett-Packard, Amstelveen, The Netherlands) equipped with a flame ionization nitrogen/phosphorous selective detector (NPD), a split/splitless injector and an autosampler (model HP 6890, Hewlett-Packard). A 25 m×0.32 mm CP Sil-13 CB capillary GC column with a film thickness of 0.25 μm was used (Chrompack, Middelburg, The Netherlands). The injector and detector temperatures were 250 and 275°C, respectively. Split injection with a split ratio of 1:5 was employed. Helium was used as carrier gas with a flow-rate of 3 ml/min. Detector gases were hydrogen (3 ml/min), air (90 ml/min) and make-up (helium, 25 ml/min). The oven temperature program started at 100°C and was maintained at this temperature for 1 min, thereafter the temperature was ramped at 60°C/min to 190°C, was maintained at 190°C for 3 min and was finally ramped to 280°C at 40°C/min and maintained at 280°C for 2.25 min. Total run-time was 10 min. Data were recorded and processed with the ChromQuest system (Thermo Separation Products, Fremont, CA, USA).

MS measurements were performed on a Finnigan Mat GCQ GC–MS combination with an A200S autosampler (Thermo Separation Products). A 30 m×0.25 mm DB-5 MS column (J&W Scientific, Folsom, CA, USA) with a film thickness of 0.25 μm was used. Splitless injection was employed with helium as carrier gas at a constant linear velocity of 40 cm/s. A Finnigan model 119418A temperature programmable injector (PTV) was used (Thermo Separation Products) which was maintained at 250°C for isothermal injection or maintained at 100°C until 1 min after injection (simultaneously with closing of the split valve) and ramped to 250°C in order to remove excess solvents. The oven temperature was programmed with 100°C for 2 min, increased at 40°C/min to 250°C at which it was maintained for 5 min. The transferline was maintained at 275°C. Measurements were performed in the positive electron impact mode (EI+/MS) with an electron energy of 70 eV, positive chemical ioniziation mode (CI+/MS) or negative chemical ionization mode (CI−/MS). Chemical ionization was performed with methane as reagent gas. The mass detector was equipped with an ion trap which allowed MS/MS measurements both in the EI and CI mode.

### 2.3. Sample pre-treatment

Blood samples were collected in heparinized tubes and centrifuged immediately at 4°C for 3 min at 1500 *g*. Plasma was collected and stored at −70°C until analysis. A 500-μl volume of plasma was diluted with 50 μl water whereafter 25 μl of a 100-μg/ml solution of trofosfamide in water were added as internal standard. Of this sample, 500 μl were applied to an OASIS HLB extraction cartridge preconditioned with 1.0 ml methanol and 2.0 ml water. The columns were washed with 1.0 ml water and dried under vacuum. Elution was performed with 1.0 ml of methanol and the organic phase was evaporated under a gentle stream of nitrogen at 40°C. Samples were reconstituted in 100 μl of methanol.

MS was tested as a detection technique for both underivatized 2-DCECP with the sample pre-treatment as described and for determination of the TFAA derivative of 2-DCECP. The TFAA derivative was prepared by reconstitution of the dry samples obtained after the SPE procedure in 100 μl ethyl acetate and 100 μl of TFAA were added subsequently. The samples were incubated for 1 h at 70°C whereafter the samples were evaporated to dryness under a gentle stream of nitrogen at 40°C and reconstituted in 100 μl of ethyl acetate.

In a final step, the assay was carried out with the

use of an automated SPE system (ASPEC XL, Gilson Medical Electronics, Middleton, WI, USA). The solid-phase procedure was similar to that described, except that to the mixture of plasma, internal standard and water (575 μl), a 600-μl volume of water was added whereafter 1 ml of this mixture was submitted to the extraction cartridges.

### 2.4. Calibration

Aliquots of drug-free human plasma (500 μl) were spiked with 50 μl of solutions of 2-DCECP in water resulting in final concentrations of 5, 10, 50, 100, 500, 1000 and 5000 ng/ml in plasma. At each calibration level, samples were prepared in duplicate. These samples were subsequently processed as described for the patient samples (see Section 2.11).

### 2.5. Validation

A three-run validation of the following parameters was performed: accuracy, within-day and between-day precision, linearity, selectivity and specificity, extraction efficiency, stability, limit of detection (LOD) and lower limit of quantitation (LLQ). Hereafter, the use of automated SPE was evaluated with a single-run validation. In this run, accuracy, precision and linearity were evaluated.

### 2.6. Extraction recovery

The extraction efficiency was determined at 2-DCECP concentrations of 100 and 1000 ng/ml and a trofosfamide concentration of 5000 ng/ml by comparing processed spiked plasma samples with unprocessed samples in methanol. To all samples, diphenylamine was added (final concentration 10 μg/ml) as external standard. The extraction recovery was calculated by comparing the ratio of the peak area of 2-DCECP or trofosfamide and diphenylamine in processed and unprocessed samples. Measurements were performed in quadruplicate.

### 2.7. Accuracy, precision and linearity

Quality control samples were prepared by spiking 500 μl aliquots of plasma with 50 μl of 2-DCECP solutions resulting in final concentrations of 5, 10, 100, 1000 and 5000 ng/ml in plasma. Five replicates of each level were analyzed together with a calibration curve in three consecutive runs. The linearity of the three calibration curves was tested with the *F*-test for lack of fit [15] with a weight factor of $1/(\text{conc.})^2$ in order to avoid bias in favour of the high standards. 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 were defined with Eqs. (1) and (2), respectively, where $n$ is the number of replicates.

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

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

For the single-run validation of the automated SPE system, the relative standard deviation of the five replicates of each quality control level was calculated as measure of precision.

### 2.8. Specificity and selectivity

Possible interference from endogenous compounds was investigated by the analysis of six different blank plasma samples. CP and all its known metabolites were investigated for interference with the analytical methods: 4-ketoCP, carboxyphosphamide, 4-hydroxyCP, phosphoramide mustard and didechloroethylCP. Carboplatin, granisetron, acetaminophen, temazepam, thioTEPA and its metabolite TEPA, dexamethasone, oxazepam and sodium 2-mercaptoethane sulphonate (MESNA) are frequently co-administered in CP-based regimens and were tested for interference. Interference was tested by spiking blank plasma with solutions of these compounds followed by sample pre-treatment and analysis. All compounds were tested at a final concen-

tration of 20 μg/ml which is equal to or exceeds each clinical relevant concentration.

### 2.9. Lower limit of quantification (LLQ) and limit of detection (LOD)

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 quantification was set at 5000 ng/ml.

### 2.10. Stability

The stability in plasma was investigated for samples kept at −70°C for 1 month including three freeze–thaw cycles. All conditions were tested at concentrations of 100 and 1000 ng/ml in duplicate. Samples were analysed together with a freshly prepared calibration curve.

Stability in the autosampler 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.

### 2.11. Patient samples

In order to demonstrate the applicability of the assay, blood samples were collected from patients treated with CP (1000 mg/m$^2$/day) in a 1-h infusion for 4 consecutive days in combination with thioTEPA (80 mg/m$^2$/day) and carboplatin (265 mg/m$^2$/day) [16].

## 3. Results and discussion

### 3.1. Chromatography and detection

The analyte was identified both in calibration and in patient samples with GC–EI+/MS and GC–CI+/MS. With EI+/MS, a base peak at *m/z* 149 was found, which corresponds to the $[M–CH_2Cl]^+$ ion. With CI+/MS, a base peak at *m/z* 199 with the chlorine isotope at *m/z* 201 was found, which corresponds to the $[MH]^+$ ion.

2-DCECP has a more hydrophilic character than its parent compound. Therefore, firstly, a stationary phase of the capillary column (CP sil 8 CB) suitable for the separation of polar compounds was selected. With this capillary column, however, thioTEPA had exactly the same retention time as 2-DCECP. As thioTEPA is very frequently used in combination with CP separation was required. Separation was accomplished with the more hydrophobic stationary phase of the CP sil 13 CB column. This resulted in minor peak broadening of 2-DCECP with no influence on the analytical performance of the assay.

Another solution for the interference by thioTEPA was considered by the use of MS as detection technique. In plasma samples in the EI+ mode, however, a relatively high background signal was found at the *m/z* ratio of 149 (base peak), which resulted in low sensitivity. The use of EI+/MS/MS did not improve the sensitivity of the assay sufficiently. Therefore, CI+/MS/MS was tested and this resulted in a LOD of approximately 100 ng/ml, which was still higher than the LOD with NPD detection (1 ng/ml). In order to improve the sensitivity with MS detection, 2-DCECP was derivatized with trifluoroacetic anhydride (TFAA) as described by Momerency et al. [7]. With TFAA, the *N*,*N*′-bis-trifluoroacetyl derivative of 2-DCECP was formed. The six fluorine atoms introduced allowed the use of CI−/MS/MS and the sensitivity was increased dramatically (LOD approximately 500 pg/ml), which is comparable to the sensitivity of a previous published assay with TFAA derivatization [7]. However, the derivative rapidly degraded in the autosampler vials with a half-life of 2–6 h at ambient temperature. Hydrolysis catalyzed by trifluoroacetic acid (TFA) was considered as cause, but removal of TFA by prolongation of the evaporation step did not increase the stability. Furthermore, the performance of the capillary column rapidly decreased possibly also caused by TFA. The PTV injector on the GC–MS system allowed the injection of the derivatization mixture (TFAA:ethyl acetate) by venting the solvents at a temperature above the boiling temperature of these solvents but below the boiling point of the analyte. By rapidly increasing the temperature of the injector, the analyte was, thereafter, introduced onto the column. However, even in the derivatization mixture the derivative proved unstable.

Therefore, the described GC–NPD system without derivatization was considered an acceptable compromise between sensitivity and applicability of the method. Split injection (1:5) was used since it showed far better reproducibility with an equal LOD as splitless injection.

Fig. 2 shows chromatograms of a sample collected before infusion and 1 h after the end of a 1-h infusion of 1000 mg/m$^2$ CP. 2-DCECP and trofosfamide eluted with retention times of 4.3 and 8.6 min, respectively.

### 3.2. Sample pre-treatment

Different types of SPE columns were tested for the extraction of 2-DCECP from plasma. No retention was observed on $C_2$, silica or cyano cartridges. Good recoveries were obtained with $C_{18}$, the OASIS HLB cartridges and phenyl cartridges. The best reproducibility was obtained with the OASIS HLB columns and, therefore, these were used for the further development of the method.

Several structural analogues of 2-DCECP were tested as internal standard. Ifosfamide and 2-dechloroethylifosfamide did not improve the performance of the system mainly due to irreproducible behaviour in the GC system. Trofosfamide showed a good recovery during the sample pre-treatment and proved to be a suitable internal standard.

The automated SPE system was subsequently tested for sample pre-treatment. Recovery with this system was increased by the addition of 600 μl of water to the plasma samples before the introduction of the sample onto the extraction cartridge. The use of this automated SPE system resulted in a less labor-intensive method.

### 3.3. Extraction recovery

Diphenylamine, used as external standard for the determination of the extraction recovery, had a retention time of 4.6 min. The recoveries of 2-DCECP and trofosfamide are shown in Table 1. The extraction recovery exceeded 80%, which was higher than or comparable to previous published SPE procedures [7,11,13].

### 3.4. Accuracy, precision and linearity

Table 2 shows details of the calibration curves of the three-run validation and of the single-run validation of the automated SPE system. All calibration curves were linear in the concentration range of 5–5000 ng/ml as demonstrated with the *F*-test for

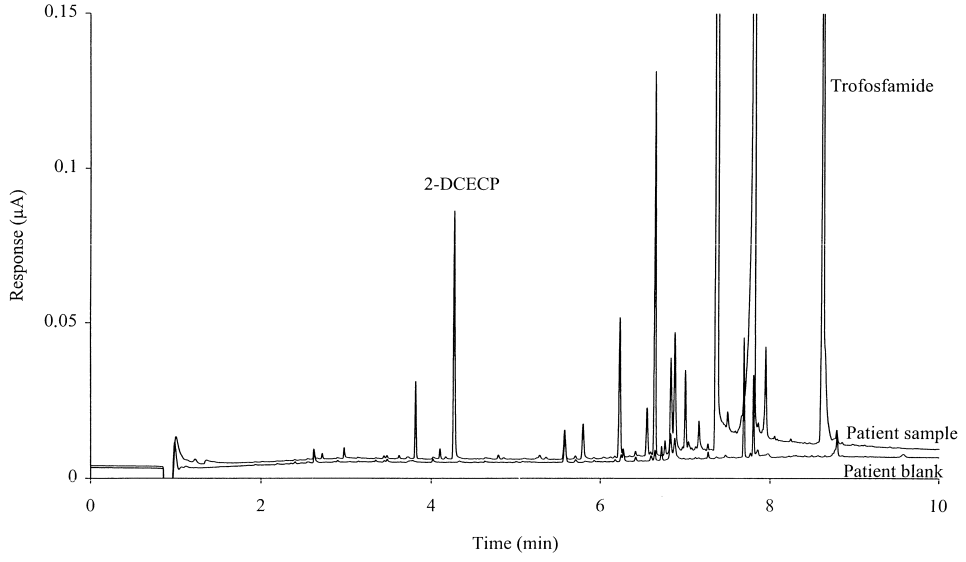


Fig. 2. Chromatograms of samples collected before and 1 h after the end of a 1-h infusion of CP (1000 mg/m$^2$) in combination with carboplatin (265 mg/m$^2$) and thioTEPA (40 mg/m$^2$). 2-DCECP concentration was 468 ng/ml.

Table 1
Extraction recovery of 2-DCECP and trofosfamide[a]

| Compound | Concentration (ng/ml) | Extraction recovery (%) | R.S.D. (%) | *n* |
|---|---|---|---|---|
| 2-DCECP | 100 | 87.2 | 7.4 | 3 |
| 2-DCECP | 1000 | 87.0 | 10.8 | 3 |
| Trofosfamide | 5000 | 85.6 | 12.0 | 6 |

[a] R.S.D., relative standard deviation; *n*, number of replicates.

Table 2
Details of the calibration curves of the three-run validation and of the single-run validation of the automated SPE system

| Validation run | Correlation coefficient (*r*) | Slope (S.E.[a]) | Intercept (S.E.) | *P*-value of lack-of-fit test | Number of samples in calibration curve |
|---|---|---|---|---|---|
| 1 | 0.998 | $3.0\times10^{-4}$ ($4.7\times10^{-6}$) | 0.34 (0.055) | 0.27 | 14 |
| 2 | 0.999 | $2.9\times10^{-4}$ ($3.5\times10^{-6}$) | 0.20 (0.039) | 0.23 | 14 |
| 3 | 0.998 | $3.0\times10^{-4}$ ($5.6\times10^{-6}$) | −0.048 (0.066) | 0.58 | 13 |
| Automated SPE system | 0.998 | $2.9\times10^{-4}$ ($4.9\times10^{-6}$) | 0.050 (0.057) | 0.70 | 14 |

[a] S.E., standard error.

Lack of Fit. The correlation coefficient (*r*) was higher than 0.998 for all calibration curves. The accuracy, within-day and between-day precisions are shown in Table 3. For all concentration levels, the accuracies and precisions were less than 11%. The 95% confidence interval of the accuracy at concentrations of 100 and 5000 ng/ml did not include 100%, but the deviation from the nominal concentration at these levels was very small.

Subsequently, the use of the automated SPE system was validated. Both the absolute signals of 2-DCECP and trofosfamide and the ratio of these two were comparable to the other validation results. The ratio of the slope of this calibration curve and the mean slope of the three calibration curves obtained without the use of the automated system was 99.2%. The calibration curve was also linear over the concentration range of 5–5000 ng/ml. The accuracy and precision of the single-run validation of this procedure are shown in Table 4. This automated system, thus, resulted in very good reproducibility with precisions of less than 2% over the complete concentration range.

### 3.5. Selectivity and specificity

Analysis of plasma samples obtained from six different individuals did not show interference of endogenous compounds. ThioTEPA and its main metabolite TEPA were identified in the chromatogram at 4.1 and 3.8 min, respectively. In the injection system, CP degrades into an internal cyclization product of CP [9]. Both this product (7.4 min) and parent CP (7.8 min) were detected in the chromatogram. These compounds did not interfere with the analysis. It was, however, impossible to

Table 3
Accuracy and precision of the determination of 2-DCECP

| Nominal concentration (ng/ml) | Within-day precision (%) | Between-day precision (%) | Accuracy (%) | 95% Confidence interval |
|---|---|---|---|---|
| 5 | 10.3 | 3.7 | 101.1 | 95.0–107.1 |
| 10 | 4.6 | [a] | 101.1 | 98.6–103.6 |
| 100 | 3.3 | [a] | 103.0 | 101.2–104.9 |
| 1000 | 2.4 | 0.15 | 101.6 | 99.5–103.7 |
| 5000 | 3.1 | 4.0 | 96.2 | 93.8–98.7 |

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

Table 4
Accuracy and precision of the determination of 2-DCECP with the use of an automated solid-phase extraction system (ASPEC)

| Nominal concentration (ng/ml) | Precision[a] (%) | Accuracy (%) | 95% Confidence interval |
|---|---|---|---|
| 5 | 1.2 | 101.0 | 97.7–104.3 |
| 10 | 2.0 | 93.1 | 87.9–98.3 |
| 100 | 2.0 | 99.4 | 93.9–104.8 |
| 1000 | 0.94 | 103.8 | 101.1–106.5 |
| 5000 | 0.89 | 102.8 | 100.3–105.4 |

[a] Calculated as relative standard deviation of five replicates at each level.

quantify CP, thioTEPA or TEPA simultaneously in the samples, due to the very large difference in observed concentrations and the irreproducible behaviour of thioTEPA and TEPA during the evaporation of the organic phase as described previously [9]. Peaks originating from caffeine (6.5 min), acetaminophen (5.2 min) and 4-ketocyclophosphamide (3.0 min) were also identified in the chromatogram but did not interfere with the analysis. All other compounds tested were not detected.

### *3.6. Lower limit of quantification (LLQ) and limit of detection (LOD)*

The LOD of the assay was 1 ng/ml with a signal-to-noise ratio of 3. The LLQ was 5 ng/ml with an accuracy and precision as shown in Table 2. This LLQ was much lower compared to previous published assays with liquid–liquid extraction without derivatization [9,12] and GC–MS assays with determination of the silyl and trifluoroacetyl derivative [8,11] (50–250 ng/ml). The LLQ was comparable to assays employing LC–MS of the underivatized compound or to a GC–MS assay of the trifluoroacetylderivative (15 and 1 ng/ml, respectively) [7,13].

### *3.7. Stability*

In plasma, 2-DCECP was stable for at least a month when kept at −70°C. Recoveries after 1 month at −70°C were 107 and 104%, for 100 and 1000 ng/ml, respectively. The recovery of 2-DCECP in processed samples after storage in the autosampler for 36 h at ambient temperature was 101.2%.

### *3.8. Analysis of patient samples*

Fig. 3 shows concentration–time curves of two patients treated with CP (1000 mg/m$^2$) in a 1-h infusion in combination with carboplatin and thioTEPA. 2-DCECP showed accumulation during treatment and therefore a considerable amount of this metabolite was still present before the next daily infusion. Because of the sensitivity of the assay, the described method can be used for the analysis of samples from patients treated with conventional or high-dose CP.

## 4. Conclusion

A sensitive and simple assay of the CP metabolite 2-DCECP has been developed and validated. Automation of the assay using an automated SPE system resulted in a less labor-intensive method with excellent reproducibility. The assays meet all current requirements for bioanalytical methods and can be used in pharmacokinetic studies of CP.

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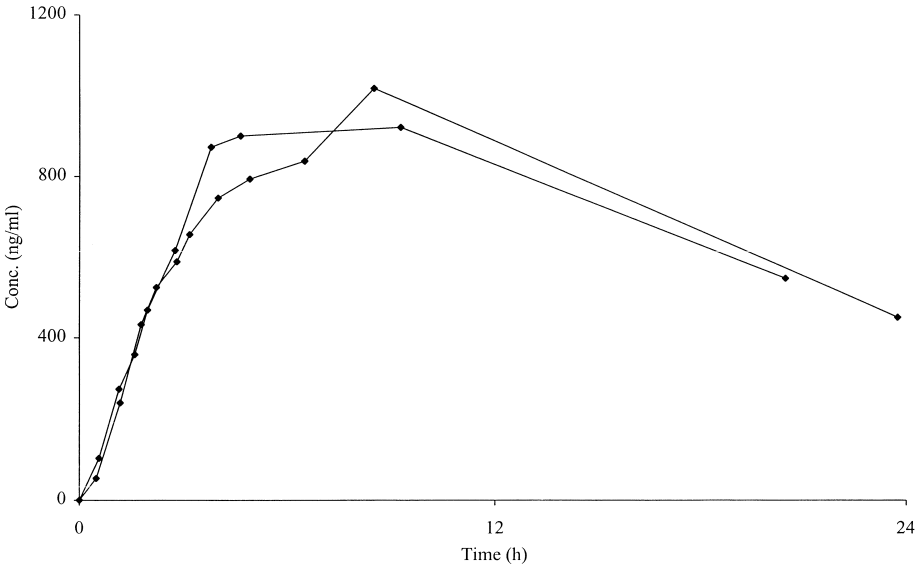


Fig. 3. Concentration–time curves of 2-DCECP of two patients treated with CP (1000 mg/m$^2$) in combination with thioTEPA and carboplatin.

## References

[1] E. van der Wall, J.H. Beijnen, S. Rodenhuis, Cancer Treat. Rev. 21 (1995) 105.
[2] A.D.R. Huitema, K. Doesburg Smits, R.A.A. Mathôt, J.H.M. Schellens, S. Rodenhuis, J.H. Beijnen, Anti-Cancer Drugs 11 (2000) 515.
[3] T. Kerbusch, J. de Kraker, H.J. Keizer, J.W.G. van Putten, H.J.M. Groen, R.L.H. Jansen, J.H.M. Schellens, J.H. Beijnen, Clin. Pharmacokinet., in press.
[4] D. Busse, F.W. Busch, F. Bohnenstengel, M. Eichelbaum, P. Fischer, J. Opalinska, K. Schumacher, E. Schweizer, H.K. Kroemer, J. Clin. Oncol. 15 (1997) 1885.
[5] A.D.R. Huitema, T. Kerbusch, M.M. Tibben, S. Rodenhuis, J.H. Beijnen, Cancer Chemother. Pharmacol. 46 (2000) 119.
[6] S. Ren, T.F. Kalhorn, G.B. McDonald, C. Anasetti, F.R. Appelbaum, J.T. Slattery, Clin. Pharmacol. Ther. 64 (1998) 289.
[7] G. Momerency, K. Van Cauwenberghe, P.H.Th.J. Slee, A.T. Van Oosterom, E.A. De Bruijn, Biol. Mass Spectrom. 23 (1994) 149.
[8] T.F. Kalhorn, S. Ren, W.N. Howald, R.F. Lawrence, J.T. Slattery, J. Chromatogr. B 732 (1999) 287.
[9] A.D.R. Huitema, M.M. Tibben, T. Kerbusch, J.W. Zwikker, S. Rodenhuis, J.H. Beijnen, J. Chromatogr. B 716 (1998) 177.
[10] F. Bohnenstengel, S. Johnson, U. Hofman, M. Eichelbaum, H.K. Kroemer, J. Chromatogr. B 672 (1995) 271.
[11] J.J.H. Wang, K.K. Chan, J. Chromatogr. B 674 (1995) 205.
[12] G.P. Kaijser, J.H. Beijnen, A. Bult, G. Wiese, J. de Kraker, H.J. Keizer, W.J. Underberg, J. Chromatogr. 583 (1992) 175.
[13] F. Baumann, C. Lorenz, U. Jaehde, R. Preiss, J. Chromatogr. B 729 (1999) 297.
[14] M.J. van Maanen, R.D. van Ooijen, J.W. Zwikker, A.D.R. Huitema, S. Rodenhuis, J.H. Beijnen, J. Chromatogr. B 719 (1998) 103.
[15] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Micotte, L. Kaufman, Chemometrics: A Textbook, in: Elsevier, New York, 1988, p. 84.
[16] S. Rodenhuis, A. Westermann, M.J. Holtkamp, W.J. Nooijen, J.W. Baars, E. van der Wall, I.C.E. Slaper-Cortenbach, J.H. Schornagel, J. Clin. Oncol. 14 (1996) 1473.