ELSEVIER

Journal of Chromatography B, 742 (2000) 335–343

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of thioTEPA, TEPA and a novel, recently identified thioTEPA metabolite, monochloroTEPA, in urine using capillary gas chromatography

M.J. van Maanen[a,b,*], K. Doesburg Smits[a], J.H. Beijnen[a,b]

[a]*Utrecht University, Department of Pharmaceutical Analysis, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[b]*The Netherlands Cancer Institute/Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

Received 18 August 1999; received in revised form 3 March 2000; accepted 8 March 2000

Abstract

An assay for the simultaneous quantitative determination of thioTEPA, TEPA and the recently identified metabolite *N*,*N*′-diethylene-*N*″-2-chloroethylphosphoramide (monochloroTEPA) in human urine has been developed. MonochloroTEPA was synthesized by incubation of TEPA with sodium chloride at pH 8. Thus, with this assay monochloroTEPA is quantified as TEPA equivalents. Analysis of the three analytes in urine was performed using gas chromatography with selective nitrogen–phosphorous detection after extraction with a mixture of 1-propanol and chloroform from urine samples. Diphenylamine was used as internal standard. Recoveries ranged between 70 and 100% and both accuracy and precision were less than 15%. Linearity was accomplished in the range of 25–2500 ng/ml for monochloroTEPA and 25–5000 ng/ml for thioTEPA and TEPA. MonochloroTEPA proved to be stable in urine for at least 4 weeks at −80°C. ThioTEPA, TEPA and monochloroTEPA cummulative urinary excretion from two patients treated with thioTEPA are presented demonstrating the applicability of the assay for clinical samples and that the excreted amount of monochloroTEPA exceeded that of thioTEPA on day 2 to 5 of urine collection.

Keywords: *N*,*N*′,*N*″-Triethylenethiophosphoramide; *N*,*N*′,*N*″-Triethylenephosphoramide; *N*,*N*′-Diethylene-*N*″-2-chloroethylphosphoramide

1. Introduction

The alkylating agent *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA) has been applied in cancer therapy now for more than 40 years [1]. ThioTEPA has recently been employed in high-dose combination regimens for breast cancer, ovarian cancer and other solid tumors, because of its broad spectrum anti-tumor activity [2].

TEPA (*N*,*N*′,*N*″-triethylenephosphoramide) was the first reported metabolite of thioTEPA, and is formed in the liver after oxidative desulfuration [3]. TEPA rapidly appears in plasma after thioTEPA infusion and has a half-life 2–7-times longer than thioTEPA [4,5].

In a recent study a new metabolite of thioTEPA with alkylating capacity was identified, the monochloro adduct of TEPA, *N*,*N*′-diethylene,*N*″-2-chlo-

*Corresponding author. University of Utrecht, Department of Pharmaceutical Analysis, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands. Tel.: +31-30-2536-918; fax +31-30-2535-180.

E-mail address: m.j.vanmaanen@pharm.uu.nl (M.J. van Maanen)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00181-X

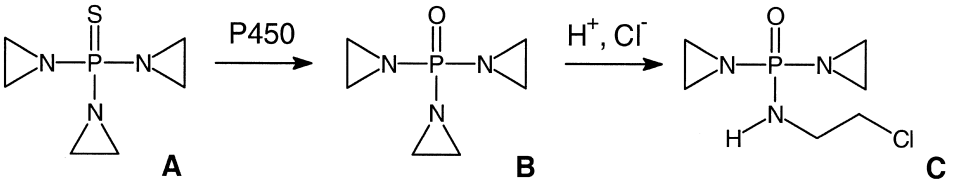

Fig. 1. Biotransformation of thioTEPA (A) to TEPA (B) and monochloroTEPA (C).

roethylphosphoramide (monochloroTEPA, Fig. 1) [6]. MonochloroTEPA was only found in urine of treated patients and not in plasma. The clinical importance of monochloroTEPA is not known yet as pharmacokinetic data of this metabolite is lacking. In order to gain more insight into this new metabolic pathway an assay was developed and validated in which monochloroTEPA is analyzed in urine together with the parent drug thioTEPA and TEPA, and which can be applied in clinical studies. Earlier methods were not useful for the analysis of monochloroTEPA [7–9].

2. Experimental

2.1. Chemicals

ThioTEPA was obtained from Cyanamid Benelux (Etten-Leur, The Netherlands). TEPA was synthesized by Dr. J.W. Zwikker (Faculty of Chemistry, Utrecht University, Utrecht, The Netherlands) as previously described [7]. Diphenylamine, used as internal standard, was purchased from Baker Analysed Reagent (Deventer, The Netherlands). All other chemicals used were of analytical grade.

2.2. Synthesis, isolation, identification and quantification of monochloroTEPA

A volume of 4 ml of a solution of 1 mg/ml TEPA in 25 m*M* phosphate buffer, pH 8 and 1 *M* sodium chloride was incubated for 2 h at 80°C. The solution was then evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted in 600 μl water. For isolation purposes four times a volume of 100 μl was injected into the high-performance liquid chromatography (HPLC) system with UV detection at 205 nm. Separation was performed on a LiChrospher 100 RP-18 (5 μm) column (125×4 mm, Merck, Darmstadt, Germany). A mobile phase of 2.5% (v/v) acetonitrile in water was used and was delivered at a flow-rate of 1 ml/min. The eluate was collected between 10 and 16 ml and evaporated to complete dryness. The identity and purity of the reaction product was established by gas chromatography–mass spectrometry (GC–MS). The yield was too small to allow an accurate absolute analysis, therefore the analyte was quantified in stock solutions with GC, using TEPA as reference.

2.3. Instrumentation

A gas chromatograph (HRGC 5300, Carlo Erba, Milan, Italy) equipped with a split/splitless injector and thermionic N/P-selective detector was used. Separation was achieved using a 25 m×0.32 mm CP Sil-8 CB capillary column (Chrompack, Middelburg, The Netherlands) with a film thickness of 0.25 μm. The oven temperature was programmed as follows: starting temperature 140°C, raised to 180°C at 5°C/min after which it was raised to 300°C with 15°C/min and kept at that temperature for 2 min. The injector was kept at 250°C using a split ratio of 1:10 and the detector temperature was 300°C. Helium was used as carrier gas at a flow-rate of 3 ml/min. Gas flow-rates were: hydrogen 4 ml/min; air 160 ml/min and make-up 13 ml/min. The current was set at 2.77 A and the voltage at 3.5 V.

GC–MS measurements were performed on a Jeol JMS-AX 505W (Jeol, Tokyo, Japan). Separation was achieved on a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA, USA) using a 25 m×0.32 mm CP Sil-8 CB capillary column (Chrompack) with a film thickness of 0.25 μm. The oven temperature was programmed as follows: starting temperature 80°C, raised to 200°C at 10°C/min. The

temperature was kept at 200°C for 2 min after which it was raised to 300°C at 25°C/min and held at that temperature for 1 min. A cold-on-column injector was used. MS measurements were performed in the electron impact (EI) positive mode. The ionization current and ion multiplier were set at 100 μA and 1.4 kV, respectively.

2.4. Sample pretreatment

Samples of 500 μl urine were transferred to polypropylene microcentrifuge tubes (Brand, Wertheim, Germany). Next, 20 μl of a 125 μg/ml diphenylamine solution in methanol and 700 μl of a 25% (v/v) solution of 1-propanol in chloroform were added, the mixture vortex-mixed for 15 s and centrifuged for 3 min at 1100 *g*. The aqueous layer was discarded and the organic layer transferred to a 0.7-ml amber glass vial (Chromacol, Trumbull, USA), and evaporated under a nitrogen stream at room temperature to approximately 20 μl. Next, 30 μl methanol was added and 1 μl of the resulting solution was injected into the chromatograph.

2.5. Calibration

Calibration samples containing 500 μl drug-free urine spiked with thioTEPA, TEPA and monochloroTEPA in the appropriate drug concentrations. An aliquot of 20 μl of a 125 μg/ml diphenylamine solution in methanol and 700 μl of a 25% (v/v) solution of 1-propanol in chloroform were added. The mixture was further treated as described in Section 2.4.

2.6. Validation

A three-run validation was completed for the quantitative determination of thioTEPA, TEPA and monochloroTEPA in urine. The following parameters were evaluated: specificity and selectivity, linearity, lower limit of quantification (LLQ), accuracy, within- and between-run precision, recovery and stability.

2.7. Specificity and selectivity

Urine samples of six different healthy volunteers and two cancer patients were analyzed to check if endogenous components co-eluted with thioTEPA, TEPA and monochloroTEPA. Interference of co-medicated drugs with the analysis of thioTEPA, TEPA and monochloroTEPA was also checked.

2.8. Linearity

2.8.1. Lack-of-fit test

The calibration curve ranged from 25 to 2500 ng/ml for monochloroTEPA and 25–5000 ng/ml for thioTEPA and TEPA in urine and was prepared from eight calibration standards of which the lowest concentration is the LLQ. Samples were analyzed in singular and the lack-of-fit test (LOF) [10] was used to evaluate the linearity of the calibration curve. The F_{LOF} was calculated by analysis of variance (ANOVA). The squared sum of residuals (SS_r, calculated with regression analysis) is divided into a pure error sum of squares (SS_{pe}) and a sum of squares due to lack of fit (SS_{LOF}). F_{LOF} is calculated by Eq. (1):

$$F_{LOF} = [(SS_r - SS_{pe})/(df_r - df_{pe})]/(SS_{pe}/df_{pe}) \quad (1)$$

in which df represents the degrees of freedom. If F_{LOF} is less than the tabulated critical value than a linear relationship was obtained.

2.8.2. Translational bias and rotational bias

To test if the method is subjected to translational or rotational bias the Student's *t*-test was applied [11] to the following linear model (Eq. (2)):

$$C_m = \alpha + \beta C_n + \epsilon \quad (2)$$

in which C_m is the measured concentration, C_n is the nominal concentration, α is the intercept, β is the slope and ϵ is the random measurement error. For the estimates of α and β (a, b) t_a and t_b are calculated by Eq. (3):

$$t_a = a/\text{S.E.}(a) \text{ and } t_b = (b-1)/\text{S.E.}(b) \quad (3)$$

in which S.E. is the standard error. If t_a and t_b are less than the tabulated critical value than $\alpha \approx 0$ and $\beta \approx 1$, indicating that there is no significant bias.

2.8.3. Relative concentration residuals

Concentrations were back calculated from the peak areas and the relative concentration residuals (RCRs) were calculated from Eq. (4):

$$\%\,\mathrm{RCR} = 100(\mathrm{RC} - \mathrm{NC})/\mathrm{NC} \quad (4)$$

in which RC is the interpolated concentration and NC is the nominal concentration.

2.9. Accuracy and precision

Quality control samples containing 25, 100, 1000 and 2500 ng/ml monochloroTEPA and 25, 100, 2500 and 5000 ng/ml thioTEPA and TEPA in urine were prepared to determine the accuracy and precision. Five replicates per concentration level were analyzed together with a standard curve in each run. The accuracy was calculated as the mean deviation (in percent) from the nominal concentration. The within- and between-run precisions were calculated by one-way ANOVA using the run as classification variable. The day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained from the ANOVA analysis. Within- and between-run precisions were calculated from Eqs. (5) and (6):

$$\text{Between-run precision} = 100\% \times [(\mathrm{DayMS} - \mathrm{ErrMS})/n]^{0.5}/\mathrm{GM} \quad (5)$$

$$\text{Within-run precision} = 100\% \times (\mathrm{ErrMS})^{0.5}/\mathrm{GM} \quad (6)$$

in which n is the number of replicates.

2.10. Recovery

The recoveries of the thioTEPA, TEPA and monochloroTEPA extraction from urine were calculated by dividing the slopes of the processed and non-processed calibration curves. The extraction efficiency of diphenylamine was determined by comparing the response of three processed samples with the response of three unprocessed samples at a concentration of 5 μg/ml.

2.11. Stability

The stability of monochloroTEPA has been studied at concentrations of 10, 50, 500 and 1000 ng/ml in urine over a period of 24 h at 22°C and 1 and 2 months at −80°C. The stability during two freeze–thaw cycles was studied at concentrations of 10, 50, 500 and 1000 ng/ml in urine. The stability testing of thioTEPA and TEPA was performed in previous studies [7,12].

2.12. Urinary excretion

The described method was used to analyze the cummulative excretions of thioTEPA, TEPA and monochloroTEPA from two patients treated with thioTEPA. The patients received cyclophosphamide 1000 mg/m^2, thioTEPA twice daily 40 mg/m^2 and carboplatin 265 mg/m^2 (tCTC regimen, Ref. [13]). Urine was collected during 24 h on each day of thioTEPA administration (days 1–4) and on the day after the last administration (day 5). After collection samples were stored at −80°C and analyzed within 1 month.

3. Results and discussion

3.1. Synthesis, isolation and identification of monochloroTEPA

In a previous study an attempt was made to unravel the metabolic profile of thioTEPA [6]. Urine of a patient receiving only thioTEPA and carboplatin was collected during thioTEPA administration. GC analysis after reversed-phase solid-phase extraction (SPE) and liquid–liquid extraction (LLE) with ethyl acetate and mixtures of 1-propanol with chloroform of urine samples revealed the presence of an additional peak in the gas chromatogram. This peak was identified as monochloroTEPA with GC–MS analysis.

MonochloroTEPA is not available as reference and it was, therefore, decided to synthesize the analyte in our laboratory. TEPA is capable to form chloro adducts after incubation with sodium chloride [14]. The highest yield of monochloroTEPA was obtained by incubation of TEPA in 1 *M* sodium chloride in

phosphate buffer, pH 8 at 80°C. A small amount of the dichloro adduct of TEPA was also formed during the synthesis of monochloroTEPA. Separation of monochloroTEPA from the reaction medium was accomplished with SPE, using C_{18} cartridges. The eluate fractions were separately collected and the amount monochloroTEPA was determined with GC. In all fractions, however, approximately 20% TEPA was present.

The use of reversed-phase cartridges with different sorbent material or normal-phase cartridges did not improve the separation between monochloroTEPA and TEPA. Therefore HPLC, with 2.5% (v/v) acetonitrile in water as mobile phase, was used. Fractions of 1 ml were collected, evaporated under a nitrogen stream at ambient temperature, and the residue was dissolved in methanol. GC analysis showed that monochloroTEPA eluted between 10 and 16 ml and these fractions were collected for the isolation. The collected fractions contained less than 5% TEPA.

The yield of monochloroTEPA was determined with GC, using TEPA as reference, because the amount of monochloroTEPA was too small to perform an absolute accurate determination. GC–MS was used for identification of the synthesized monochloroTEPA, showing a molecular ion at m/z 209 with an isotope at 211 (Fig. 2A). The ratio 3:1 for m/z 209 and 211 indicates the presence of a chlorine atom. The mass spectrum shows loss of chlorine

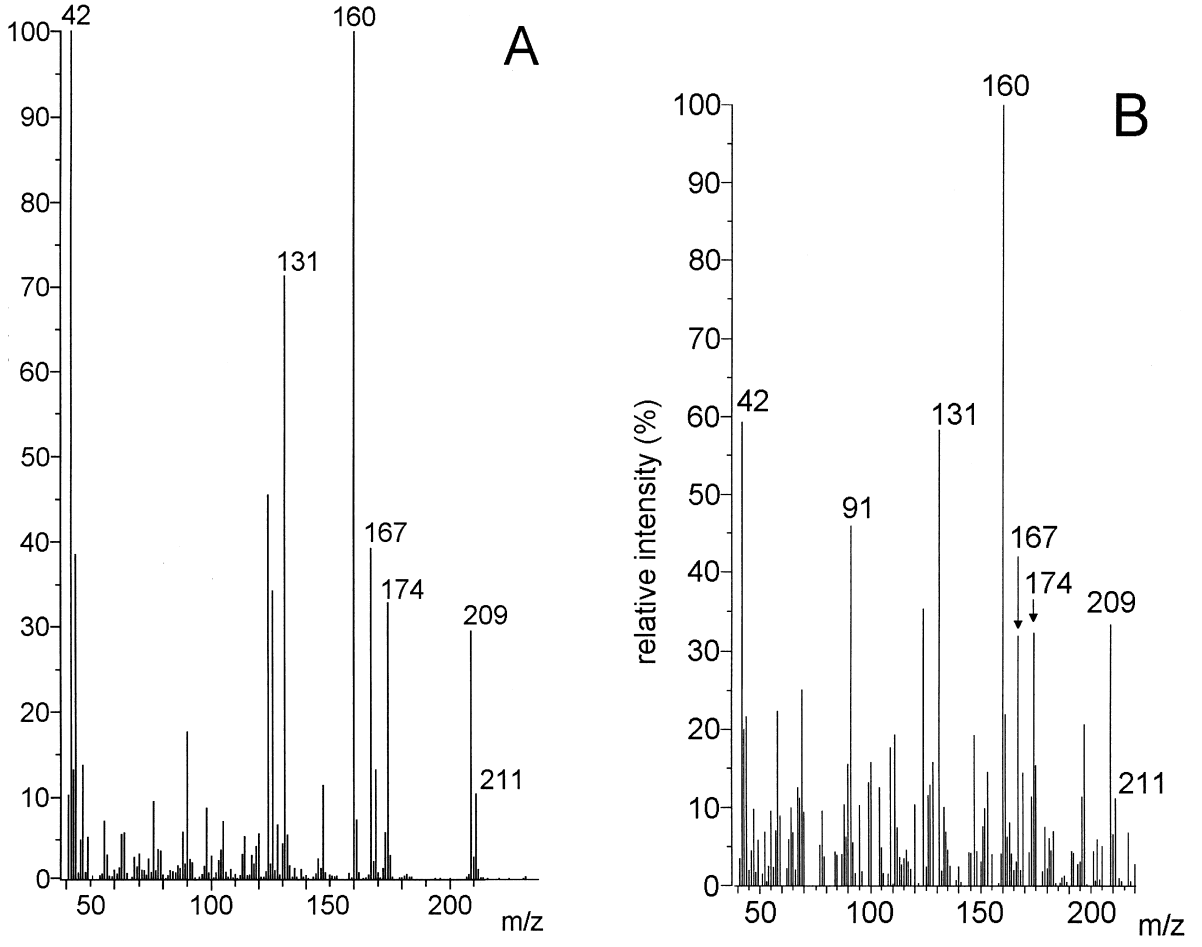

Fig. 2. GC–MS of monochloroTEPA, synthesized by incubation of TEPA with sodium chloride for 2 h in 25 m*M* phosphate buffer, pH 8 at 80°C (A) and of the metabolite of thioTEPA (monochloroTEPA) isolated from patients' urine with LLE (B).

(m/z 174; same mass as protonated TEPA), aziridine (m/z 167), chloromethyl (m/z 160), and chloroethyl (m/z 131); m/z 42 originates from an aziridine group; m/z 91 is a background signal. In Fig. 2B the mass spectrum of monochloroTEPA isolated from urine of treated patients is shown, and is identical to the synthesized product (Fig. 2A).

3.2. Extraction

The extraction of monochloroTEPA was initially performed as described for both thioTEPA and TEPA [7]. In this method thioTEPA and TEPA were extracted from biological fluids with a mixture of 25% (v/v) 1-propanol in chloroform from only 100 μl sample, using these conditions the limit of quantification (LOQ) for monochloroTEPA was 100 ng/ml. To decrease the LOQ, the amount of urine used for the extraction was increased to 500 μl to which 50 μl 1 *M* NaOH was added and extraction was performed with 700 μl 25% (v/v) 1-propanol in chloroform. The organic layer was evaporated to approximately 20 μl and 30 μl methanol was added. By these procedures the LOQ was brought to 10 ng/ml and the recovery of monochloroTEPA was 98%. Linearity was accomplished in the range of 10–1000 ng/ml, the accuracy, within- and between-run precision were all less than the acceptable 15% [15] (Table 1). Simultaneous extraction of thioTEPA, TEPA and monochloroTEPA, however, resulted in non-linear calibration curves for TEPA and monochloroTEPA, but not for thioTEPA. The presence of monochloroTEPA and TEPA in an alkaline solution might result in the formation of dimers of TEPA (Fig. 3), as described for various activated aziridines [16], and may lead to poor linearity.

If the extraction was performed without the addition of NaOH, linear calibration curves were obtained for all three analytes. The LOQ for monochloroTEPA was decreased to 25 ng/ml. Using the method described above, the recoveries of thioTEPA, TEPA and monochloroTEPA were 94% (±7.8%), 72% (±11%) and 106% (±7.8%), respectively. The recovery of diphenylamine at a concentration of 5 μg/ml was 80% (±4.0%).

Table 1
Accuracy and precision of monochloroTEPA analysis in urine

	Nominal concentration (ng/ml)			
	10	50	500	1000
Accuracy	5.0	−2.5	5.3	3.7
Within-run precision	4.9	3.4	3.7	3.1
Between-run precision	12.4	8.0	8.0	11.1

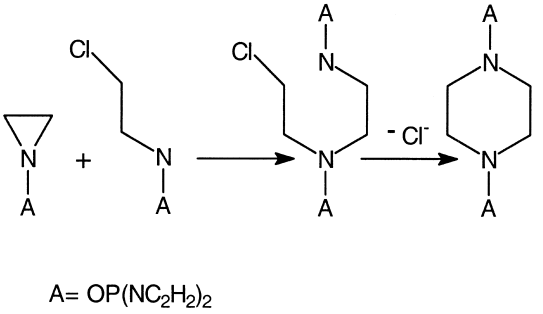

Fig. 3. Dimerization of monochloroTEPA with TEPA [16].

3.3. Chromatography

Fig. 4 shows chromatograms of a urine sample of a patient before and after treatment with thioTEPA. The concentrations of thioTEPA, TEPA and monochloroTEPA were 587, 1825 and 82 ng/ml, respectively. The chromatographic conditions provide good separation between thioTEPA, TEPA, monochloroTEPA and diphenylamine. Retention times of TEPA, thioTEPA, monochloroTEPA and diphenylamine were 3.4, 4.0, 5.1 and 4.9 min, respectively.

3.4. Linearity

Calibration curves were constructed in the range of 25–2500 ng/ml for monochloroTEPA and 25–5000 ng/ml for thioTEPA and TEPA in urine. The linearity was evaluated by the LOF test, resulting in α values >0.05 indicating a linear relationship. For all calibration curves t_a and t_b were less than the tabulated critical value, so $\alpha \approx 0$ and $\beta \approx 1$, indicating that there is no translational nor rotational bias. The RCR values at the LLQ were less than 20% and the RCR values at the other concentration levels were less than 15% and thus acceptable [15].

Fig. 4. GC chromatogram of a urine sample, collected prior to (A) and after 6 h of drug administration on day 1 (B). ThioTEPA (4.0 min), TEPA (3.4 min) and monochloroTEPA (5.1 min) concentrations were 587, 1825 and 82 ng/ml, respectively.

3.5. Accuracy and precision

In Table 2 data of the assay performance are presented for thioTEPA, TEPA and monochloro-TEPA in urine. The accuracy, within- and between-run precisions were within the acceptable criteria of $\leq$15% [15].

3.6. Specificity and selectivity

No endogenous components interfered with the determination of thioTEPA, TEPA and monochloro-TEPA, which was shown after the analysis of blank urine of six different individuals and two patients. No interference was seen of the co-medicated drugs

Table 2
Accuracy and precision of simultaneous analysis of thioTEPA, TEPA and monochloroTEPA in urine

		Nominal concentration (ng/ml)				
		25	100	1000[a]	2500	5000
ThioTEPA	Accuracy	−2.3	7.8	–	−4.1	−12
	Within-run	4.0	6.4	–	5.8	3.6
	Between-run	5.1	–[b]	–	0.6	3.0
TEPA	Accuracy	−0.7	7.6	–	0.8	−5.2
	Within-run	7.5	4.8	–	9.6	4.5
	Between-run	11	5.3	–	–	1.4
MonochloroTEPA	Accuracy	3.6	4.4	6.3	3.7	
	Within-run	9.5	5.9	9.9	7.9	
	Between-run	12	1.0	7.3	–	

[a] This concentration was not used as quality control samples for thioTEPA and TEPA.
[b] No statistical significant additional dispersion observed due to analysis between different runs.

with the determination of thioTEPA, TEPA and monochloroTEPA.

3.7. Stability

No significant decrease in concentration of monochloroTEPA in urine was observed at 4°C, 22°C and during two freeze–thaw cycles over the test periods (Table 3). The stability proved to be satisfactory during the storage at −80°C for 1 month, whereas after 2 months a decrease of 24% was observed at 1000 ng/ml.

3.8. Cummulative urinary excretion in patients

The total amount of thioTEPA, TEPA and monochloroTEPA excreted on each day of urine collection of two patients is depicted in Fig. 5. The excreted amount of monochloroTEPA, exceeded that of

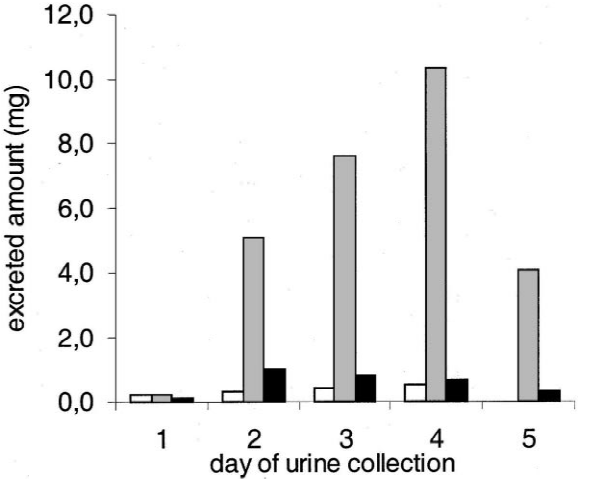

Fig. 5. Total amount of excreted thioTEPA (white bars), TEPA (dotted bars) and monochloroTEPA (calculated as TEPA equivalents, black bars) in urine of two patients treated with 40 mg/m^2 thioTEPA, two times per day, on each day of urine collection.

Table 3
Stability of monochloroTEPA in urine at 4 and 22°C after 24 h storage at −80°C during 1 and 2 months, and during two freeze–thaw cycles, concentrations calculated as percentage of the initial concentration

	Concentration (ng/ml)			
	10	50	500	1000
22°C	–	77±7.8	89±14	696±8.6
4°C	89±13	94±4.8	90±4.3	85±4.1
−80°C (1 month)	–	88±5.7	95±18	81±11
−80°C (2 months)	94±5.6	100±8.7	95±6.6	76±7.7
Two freeze–thaw cycles	102±13	98±8.9	82±4.8	86.4±13

thioTEPA on day 2 to 5 of urine collection. The excreted amount of thioTEPA, TEPA and monochloroTEPA, calculated as thioTEPA equivalents, accounted for 0.4–8.4% of the administered dose. Whereas thioTEPA is also able to form chloro adducts in aqueous solutions [17] and in acidified urine [18], the presence of chloro adducts of thioTEPA in urine was investigated, but could not be detected.

4. Conclusions

The described GC method combined with a liquid–liquid sample pretreatment procedure is suitable for the simultaneous determination of thioTEPA and its metabolites TEPA and monochloroTEPA in urine of patients treated with thioTEPA. This assay allows the analysis of thioTEPA and its metabolites in a single run after a single extraction of the sample. The presented validated method can be used for pharmacokinetic clinical studies.

References

[1] M. Sykes, D. Karnovsky, F. Phillips, J. Burchenal, Cancer 6 (1953) 142.
[2] E. van der Wall, J.H. Beijnen, S. Rodenhuis, Cancer Treat. Rev. 21 (1995) 105.
[3] L.B. Mellet, L.A. Woods, Cancer Res. 20 (1960) 524.
[4] B. Hagen, G. Neverdal, R.A. Walstad, O.G. Nilsen, Cancer Chemother. Pharmacol. 25 (1990) 257.
[5] B. Hagen, Cancer Chemother. Pharmacol. 27 (1991) 373.
[6] M.J. van Maanen, I.M. Tijhof, J.M.A. Damen, C. Versluis, J.J. Kettenes-Van den Bosch, A.J.R. Heck, S. Rodenhuis and J.H. Beijnen, Cancer Res., in press.
[7] R.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.
[8] P.J. O'Dwyer, F. LaCreta, P.F. Engstrom, R. Peter, L. Tartaglia, D. Cole, S. Litwin, J. DeVito, D. Poplack, R.J. DeLap, R.L. Comis, Cancer Res. 51 (1991) 3171.
[9] M.J. Egorin, B.E. Cohen, E.A. Kohlhepp, P.L. Gutierrez, J. Chromatogr. 343 (1985) 196.
[10] D.L. Massart, B.G.M. Vandeginste, S.N. Deming (Eds.), Chemometrics – A Textbook, Elsevier, Amsterdam, 1988, p. 80, Chapter 5.
[11] M. Thompson, Anal. Proc. 27 (1990) 142.
[12] R.J. van Maanen, R.D. van Ooijen, J.H. Beijnen, J. Chromatogr. B 698 (1997) 111.
[13] S. Rodenhuis, A. Westermann, M.J. Holtkamp, W.J. Nooijen, J.W. Baars, E. Van der Wall, I.C.M. Slaper-Cortenbach, J.H. Schornagel, J. Clin. Oncol. 14 (1996) 1473.
[14] M.J. van Maanen, I.M. Tijhof, M.J.A. Damen, J.W. Zwikker, J.H. Beijnen, Int. J. Pharm., submitted for publication.
[15] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375.
[16] O.C. Dermer, G.M. Ham (Eds.), Ethylenimine and Other Aziridines, Academic Press, New York, 1969, p. 106, Chapter 3.
[17] M.J. van Maanen, A.C. Brandt, J.M.A. Damen, J.H. Beijnen, Int. J. Pharm. 179 (1999) 55.
[18] B.E. Cohen, M.J. Egorin, M.S.B. Nayar, P.L. Gutierrez, Cancer Res. 44 (1984) 4312.