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Determination of *N,N′,N″*-triethylenethiophosphoramide and its active metabolite *N,N′,N″*-triethylenephosphoramide in plasma and urine using capillary gas chromatography

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Abstract

A sensitive assay for the determination of *N,N′,N″*-triethylenethiophosphoramide (thioTEPA) and its metabolite *N,N′,N″*-triethylenephosphoramide (TEPA) in micro-volumes human plasma and urine has been developed. ThioTEPA and TEPA were analysed using gas chromatography with selective nitrogen–phosphorus detection or mass spectrometry after extraction with a mixture of 1-propanol–chloroform from the biological matrix. Diphenylamine was used as internal standard. The limit of detection was 1.5 ng/ml for thioTEPA and 2.5 ng/ml for TEPA, using 100 μl of biological sample; recoveries ranged between 70 and 90% and both accuracy and precision were less than 10%. Linearity was accomplished in the range of 10–1000 ng/ml for plasma and 100–10 000 ng/ml for urine using thermionic nitrogen–phosphorus detection. With mass spectrometry a linear range of 100–25 000 ng/ml TEPA in plasma or urine was obtained. For thioTEPA a second-order polynomial function describes the relationship between the analyte concentration in the range of 500–25 000 ng/ml and detection response. TEPA proved to be stable in plasma and urine for at least 10 weeks at −80°C. ThioTEPA and TEPA plasma concentrations of two patients treated with thioTEPA are presented demonstrating the applicability of the assay for clinical samples.

Keywords: *N,N′,N″*-Triethylenethiophosphoramide; *N,N′,N″*-Triethylenephosphoramide

1. Introduction

N,N′,N″-Triethylenethiophosphoramide (thioTEPA, Fig. 1) is an alkylating agent, applied in cancer therapy for more than 40 years [1]. Recently

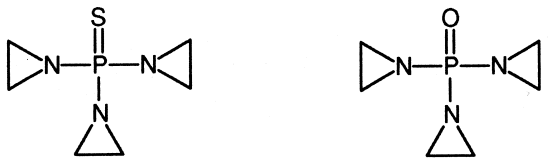

Fig. 1. Structural formulas of thioTEPA (left) and TEPA.

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thioTEPA is being employed in high dose combination regimens because of its broad spectrum of antitumor activity [2]. The appearance of the main metabolite of thioTEPA, *N*,*N*′,*N*″-triethylenephosphoramide (TEPA, Fig. 1) was first reported by Mellet and Woods [3]. TEPA is formed in the liver after oxidative desulfuration of thioTEPA [4,5] and also possesses alkylating activity. TEPA appears rapidly in plasma after thioTEPA infusion [6,7] and has a half-life time 2- to 7-times longer than thioTEPA [8,9]. Multi-cycle high-dose regimens containing thioTEPA may cause severe renal and hepatic toxicity in [10]; the role of TEPA in this is not known. Pharmacokinetic monitoring in order to individualise dosing may reduce inter-individual variability and thereby the incidence of side effects. Analytical assays used in pharmacokinetic studies of thioTEPA and TEPA in conventional dose regimens are very diverse. In some studies thioTEPA and TEPA were analysed in one run using a liquid–liquid extraction (LLE) [11] or solid-phase extraction (SPE) [6,12] as sample pre-treatment procedure followed by gas chromatography (GC). Others used two different extraction methods for thioTEPA and TEPA [7,13,14]. The sensitivity of the assays ranged between 1–5 ng/ml, using 250 μl biological material. Results of various studies are contradictory, whereas ethyl acetate is used to extract both thioTEPA and TEPA by O'Dwyer et al. [15] and Egorin et al. [16] report that TEPA cannot be extracted with ethyl acetate. The intention of this study was to develop a novel assay in which thioTEPA and TEPA are analysed in a single run using micro-volumes (100 μl) of plasma or urine. Also the use of mass spectrometry (MS) as detection mode was studied to see if a more sensitive method could be obtained.

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, University of Utrecht, Utrecht, The Netherlands). Diphenylamine, used as internal standard, was purchased from Baker Analysed Reagent (Deventer, The Netherlands). Benzene was purified by distillation from sodium/benzophenone in an atmosphere of dry nitrogen. All other chemicals used were of analytical grade unless otherwise specified and were used without purification.

2.2. Synthesis and structure identification of TEPA

The procedure for the synthesis of TEPA described by Craig and Jackson [17] was slightly modified. Thus, in an atmosphere of dry nitrogen 3.99 g (93 mmol) aziridine and 10.01 g (99 mmol) triethylamine were dissolved in 50 ml dry benzene contained in a two-necked round bottom flask equipped with a magnetic stirring bar and a drop funnel. While the flask was cooled in an ice-bath, a solution of 4.60 g (30 mmol) phosphorus oxychloride in 20 ml benzene was added dropwise with vigorous stirring. After completion of the addition (about 45 min) the cooling bath was removed and stirring was continued for 24 h. The white precipitate was removed by filtering through a glass filter (G4). The reaction flask and precipitate were thoroughly washed with benzene. Concentrating the combined filtrate and washings at a rotary evaporator, followed by removal of remaining traces of volatile compounds in an oilpump vacuum at room temperature, afforded 4.48 g of a slightly yellow oil. This was distilled in a micro-distillation apparatus yielding a main fraction of 3.53 g (68%) of a colorless oil boiling at 91–93°C/0.04 mm.

Benzene is a known human carcinogen and inhalation causes drowsiness, dizziness and unconsciousness. Long-term benzene exposure causes effects on the bone marrow and can cause anemia and leukemia. One should proceed with caution when the above described synthetic procedure is followed.

The identity and purity of the reaction product were established by nuclear magnetic resonance (NMR) and GC–MS. For GC–MS analysis a solution of 100 μg/ml TEPA in methanol was used. The ^{1}H-NMR spectrum was recorded on a Gemini 300 BB spectrometer (Varian, Palo Alto, CA, USA) at 300.1 MHz. A 12-mg sample was dissolved in 0.65 ml deuterated chloroform. The chloroform signal at 7.26 ppm was used as the reference line.

2.3. Instrumentation

A gas chromatograph (HGRC 3500, Carlo-Erba Strumentazion, Milan, Italy) equipped with a split/splitless injector and a thermionic nitrogen–phosphorus-selective detector was used. Separation was performed 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, ramped to 180°C at 5°C/min. The temperature was kept at 180°C for 2 min and ramped to 280°C at 15°C/min. The injector was kept at 250°C using a split ratio of 1:10 for plasma and 1:15 for urine, and the detector temperature was 300°C. Helium was used as carrier gas at a flow-rate of 3 ml/min. Gas flow-rates for the detector were: hydrogen 4 ml/min, air 160 ml/min and make-up 13 ml/min. The current was set at 2.71 A and the voltage at 3.5 V. MS measurements were performed on a Varian GC–MS combination (Wallnut Creek, CA, USA). GC separation was performed on a 10 m×0.25 mm DB5-MS capillary column (J&W Scientific, CA, USA) with a film thickness of 0.25 μm, using helium as carrier gas at a flow-rate of 1 ml/min. The oven temperature was programmed as follows: 2 min at 65°C, ramped to 230°C at 12°C/min, and next ramped to 280°C at 50°C/min. The temperature was kept at 280°C for 150 s. A temperature programmed injector was used, with the following program: 45 s at 80°C, ramped to 290°C at 100°C/min. The temperature was kept at 290°C for 3 min. MS measurements were performed on a Saturn-2 (Varian) operating in the positive chemical ionisation (CI) mode, with methane as reaction gas and the multiplier was set at 2 kV.

2.4. Sample pretreatment

Samples of 100 μl plasma or urine were transferred to polypropylene microcentrifuge tubes (780500, Brand, Wertheim, Germany). Then 100 μl water, 20 μl of a 10 μg/ml diphenylamine solution in methanol, 10 μl of a 1 *M* NaOH solution and 500 μl of a 10% or 25% (v/v) solution of 1-propanol in chloroform was added for plasma and urine, respectively. The mixture was vortexed for 15 s and centrifuged for 3 min at 1100 *g*. The aqueous layer and protein cake (for plasma) were discarded and the organic layer was transferred into a 0.7-ml amber glass vial (Chromacol, Trumbull, USA). The organic solvent of the plasma extraction was evaporated under a nitrogen stream at ambient temperature to near dryness. The residue was dissolved in 20 μl methanol; 1–2 μl was injected into the chromatograph. After the urine extraction the chloroform part of the chloroform–1-propanol mixture was evaporated and 1–2 μl of the remaining 1-propanol solution was injected.

2.5. Calibration

Calibration samples containing 100 μl drug free human plasma or urine were spiked with 25–100 μl of a thioTEPA and TEPA solution in water with appropriate drug concentrations. If necessary this amount was supplemented with water to a final volume of 200 μl. An aliquot of 20 μl of a 10 μg/ml or 100 μg/ml diphenylamine solution in methanol for plasma and urine, respectively and 10 μl of a 1 *M* NaOH solution were then added and 500 μl of a 10% or 25% (v/v) solution of 1-propanol in chloroform was added for plasma and urine, respectively. The mixture was further treated as described in Section 2.4.

For the GC–MS measurements a concentration of 5 μg/ml diphenylamine was used. The organic layer was transferred into a 0.5-ml Eppendorf reaction vial. The organic layer from the plasma extraction was evaporated under a nitrogen stream at ambient temperature to near dryness. The residue was dissolved in 150 μl methanol; 1–2 μl was injected into the chromatograph. After the urine extraction the chloroform part of the chloroform–1-propanol mixture was evaporated, 50 μl methanol was added and 1–2 μl of the solution was injected.

2.6. Validation

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

2.7. Specificity and selectivity

Plasma and urine samples of six different individuals were analyzed to check whether if endogenous components co-eluted with thioTEPA, TEPA of diphenylamine.

Solutions of cyclophosphamide and the selected co-medicated drugs ciprofloxacin, itraconazol, ranitidine, granisetron, dexamethason and lorazepam are also analyzed to check whether if these drugs interfered with the determination of thioTEPA, TEPA and diphenylamine.

2.8. Linearity

Calibration curves ranging from 10–1000 ng/ml in plasma and 100–10 000 ng/ml in urine were each prepared from seven calibration standards of which the lowest concentration is the LLQ. Samples were analyzed in singular and the lack of fit (LOF) test [18] was used to evaluate the linearity of the calibration curve. The F_{LOF} was calculated by analysis of variance. 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.

2.9. Limit of detection

The LOD for thioTEPA and TEPA in plasma and urine was determined at a signal-to-noise ratio of 3.

2.10. Accuracy and precision

Quality control samples containing 10, 50 and 500 ng/ml thioTEPA and TEPA in plasma and 100, 500 and 5000 ng/ml thioTEPA and TEPA in urine were prepared to determine the accuracy and precision. Five replicates of each quality control sample 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 analysis of variance (ANOVA) using the run as the classification variable. The day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained from the ANOVA analysis. Within-run and between-run precessions were calculated from Eqs. (2) and (3), respectively, where n is the number of replicates.

$$\text{Between-run precision} = 100\% \times [(\text{DayMS} - \text{ErrMS})/n]^{0.5}/\text{GM} \quad (2)$$

$$\text{Within-run precision} = 100\% \times (\text{ErrMS})^{0.5}/\text{GM} \quad (3)$$

In a previous study [19], pharmacokinetic analysis showed that C_{max} of thioTEPA in plasma ranged from 1–5 μg/ml. The maximal concentration in linear range of thioTEPA in this study appears to be 1 μg/ml. Therefore dilution steps of 10 and 100 are validated with respect to accuracy and precision. Plasma is spiked with 5 μg/ml thioTEPA and TEPA and diluted to 500 ng/ml and 50 ng/ml. Five replicates of each dilution step were analyzed together with a calibration curve in one run. Accuracy and precision are calculated from the data as described above.

2.11. Recovery

The recoveries of the thioTEPA and TEPA extraction from plasma and 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 2 μg/ml for plasma and 20 μg/ml for urine.

2.12. Stability

The stability of TEPA has been studied at concentrations of 10, 50 and 500 ng/ml in plasma and 100, 500 and 5000 ng/ml in urine during a period of 10 weeks at −80°C. Five replicates were analyzed for each concentration level.

The stability testing of thioTEPA was performed in a previous study [19].

The stability of thioTEPA and TEPA in methanol

and 1-propanol after extraction during storage in the autosampler over a 24-h time period has been studied at a concentration level of 200 ng/ml.

The stability of TEPA during two freeze–thaw cycles has been studied at concentrations of 10, 50 and 500 ng/ml in plasma and 100, 500 and 5000 ng/ml in urine. Four replicates were analyzed for each concentration level.

2.13. Pharmacokinetic case study

The described method was used to analyze thioTEPA and TEPA plasma concentrations of two patients treated with 60 mg/m^2 or 40 mg/m^2 thioTEPA. Also the thioTEPA and TEPA urine concentration of a patient treated with 40 mg/m^2 was determined. The dosing regimen of the patients was as follows: cyclophosphamide 1.0 or 1.5 g/m^2 on days 1 to 4; carboplatin 265 or 400 mg/m^2 on days 1 to 4; thioTEPA two times daily 40 or 60 mg/m^2 on days 1 to 4. Patients were sampled following their first thioTEPA administration. Blood samples were obtained before the start of the treatment, 30, 45, 60, 90, 160, 210 and 270 min after the start of the thioTEPA infusion and the next day before the start of the new infusion. Samples were centrifuged and after storage at −80°C plasmas were analyzed within two weeks. 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). Samples were stored at −80°C and analyzed within two weeks.

3. Results and discussion

3.1. Synthesis and identification of TEPA

Since various attempts to obtain TEPA as a reference for the validation of the assay where unsuccessful, TEPA was synthesized according to the method of Craig and Jackson [17]. The purity and identity of the product was tested using NMR and GC–MS. GC–MS revealed an ion with m/z ratio of 174 derived of the molecular ion. A fragment ion at $m/z = 131$ corresponds to the loss of an aziridine ring. The ^{1}H-NMR spectrum showed a single doublet at 2.18 ppm, $J_{PH} = 14.4$ Hz. TEPA was stored at −70°C under nitrogen and appears stable for at least one year.

3.2. Extraction

A single extraction method for both thioTEPA and TEPA, using micro-volumes of biological specimen has not been reported. Applied analytical methods are very diverse and results are in contradiction [6,11,15,16]. Because of this diversity a novel assay was developed to extract thioTEPA and TEPA using a single extraction method.

Extraction of thioTEPA and TEPA was initially performed according to a method used by the authors for the extraction of thioTEPA [19]. In this method, thioTEPA was extracted with ethyl acetate. Experiments showed that the recovery of TEPA was less than 25%, confirming the findings of Egorin et al. [16] and Strong et al. [13]. Poor recoveries (less than 40%) were also obtained using SPE, which was used to extract both thioTEPA and TEPA from biological samples [6,20]. Chloroform was also used to extract TEPA from biological matrices [13,16]; therefor we investigated this solvent to extract both thioTEPA and TEPA. Recoveries, however, were not satisfactory (≤60%). Next, mixtures of chloroform and alcohols with higher boiling points were tested. Recoveries of 85% for thioTEPA and 90% for TEPA were obtained at a concentration level of 200 ng/ml using a solution of 10% (v/v) 1-propanol in chloroform. The organic layer was evaporated under a stream of nitrogen at ambient temperature to near dryness. Evaporation to complete dryness must be avoided, as it will give substantial loss of thioTEPA probably due to evaporation [21]. The residue was dissolved in 20 μl methanol.

The described method was used for both plasma and urine extraction. Data showed, however, that thioTEPA and TEPA recoveries from urine were irregular. A possible explanation can be that thioTEPA and TEPA are only partially reconstituted after evaporation of the solvent to near dryness. Therefore the evaporation procedure was limited to only evaporation of the chloroform part of the organic layer. The remaining 1-propanol was injected. Results improved, but were not satisfactory for lower concentrations. The percentage of 1-propanol was raised from 10 to 25% (v/v) percent.

Again only the chloroform was evaporated and the 1-propanol was injected. This procedure resulted in reproducible results and was applied for the extraction of thioTEPA and TEPA from urine.

Using the method described above the recovery of thioTEPA was 74% (±7.6) for plasma and 82% (±11.4) for urine. Recoveries of TEPA from plasma and urine were 82% (±9.4) and 79% (±10.1), respectively. The recovery of diphenylamine at a concentration of 2 μg/ml for plasma and 20 μg/ml for urine was 81% (±7.7) and 82% (±13), respectively.

3.3. Chromatography

Chromatograms of plasma and urine samples of a patient before and after treatment with thioTEPA are shown in Fig. 2. The gas chromatograph was equipped with a selective thermionic nitrogen–phosphorus detection (TI-NPD) system. This detector was preferred over a flame ionization NPD (FI-NPD), because a previous study showed that the TI-NPD gives a better linearity and a higher sensitivity [19]. Fig. 2a shows a chromatogram of a plasma sample taken 60 min after thioTEPA infusion. In Fig. 2b the chromatogram of a urine sample collected during 24 h after thioTEPA administration is shown. The applied chromatographic method provided good separation between thioTEPA, TEPA and diphenylamine. Retention times found were 3.7 min, 4.4 min and 5.5 min for TEPA, thioTEPA and diphenylamine, respectively. The peak with a retention time of 12.8 min originates from cyclophosphamide, a cytotoxic drug co-medicated in the high-dose regimen. Measurements of thioTEPA, TEPA and diphenylamine using GC–MS also provided good separation. Retention times were 7.8, 8.2 and 9.1 min for TEPA, thioTEPA and diphenylamine, respectively. In Fig. 3 the mass spectra of thioTEPA, TEPA and diphenylamine are presented.

3.4. Validation

A complete validation was performed on the described assay using the GC equipped with the TI-NPD. The GC–TI-NPD and GC–MS systems were compared with regard to linearity and sensitivity.

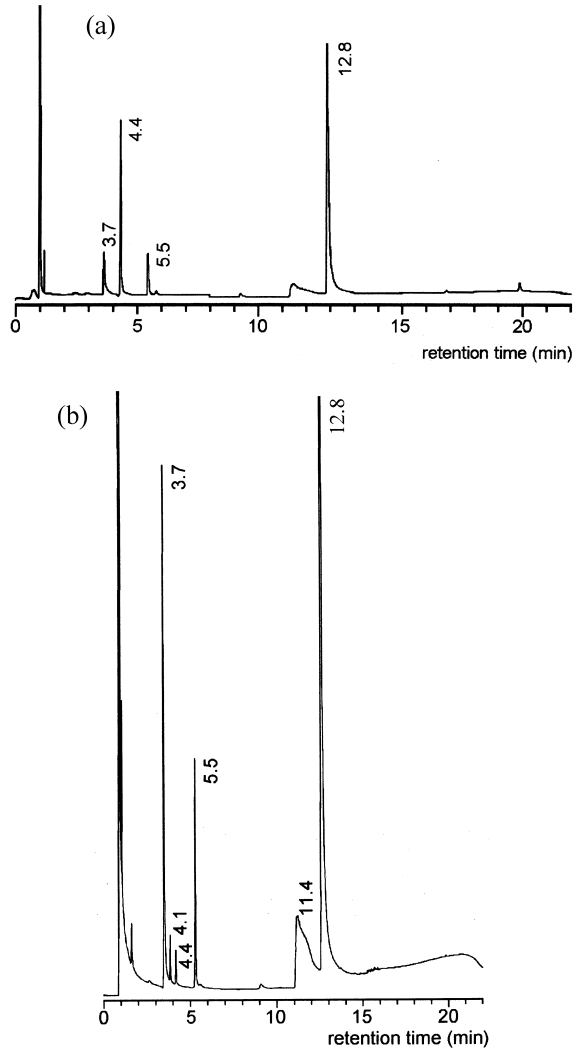

Fig. 2. (a) Patient plasma sample after treatment with thioTEPA, 90 min after administration. ThioTEPA concentration is 522 ng/ml and TEPA 310 ng/ml. Peaks corresponding to retention times of 3.7, 4.4 and 5.5 are TEPA, thioTEPA and diphenylamine, respectively. The peak eluting at 12.8 min originates from cyclophosphamide. After 8 min attenuation changed from 8 to 64. (b) Patient urine sample after treatment with thioTEPA. ThioTEPA concentration is 334 ng/ml and TEPA 10 μg/ml. Peaks corresponding to retention times of 3.7, 4.4 and 5.5 are TEPA, thioTEPA and diphenylamine, respectively. The peak eluting at 12.8 min originates from cyclophosphamide. The peak with retention time 4.1 min is also present in blank urine and thus originates from endogenous substituents.

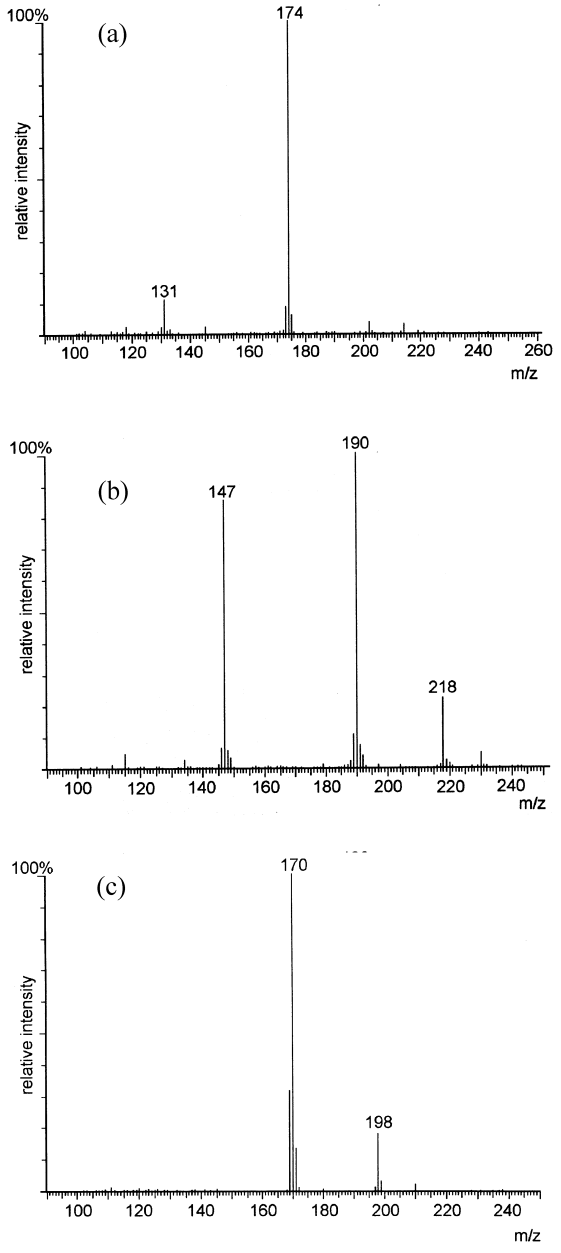

Fig. 3. (a) GC mass spectrum of TEPA in a spiked plasma sample. Fragments $m/z=174$ and $m/z=131$ originate from $[MH]^+$ and $[M-C_2H_4N]^+$, respectively. (b) GC mass spectrum of thioTEPA in a spiked plasma sample. Fragments $m/z=190$, $m/z=147$ originate from $[MH]^+$ and $[M-C_2H_4N]^+$, respectively. Fragment $m/z=218$ corresponds to $[M+C_2H_5]^+$, which is an adduct of the molecular ion and the reaction gas. (c) GC mass spectrum of diphenylamine in a spiked plasma sample. Fragment $m/z=170$ originates from $[MH]^+$. Fragment $m/z=198$ corresponds with $[M+C_2H_5]^+$, which is an adduct of the molecular ion and the reaction gas.

3.5. Linearity

Calibration curves were constructed in the range of 10–1000 ng/ml for plasma and 100–10 000 ng/ml for urine. To avoid biasing the calibration curves in favor of the high standards, weighting factors $1/x$ and $1/x^2$ were introduced in which x represents the thioTEPA or TEPA concentration [22,23]. Correlation coefficients (r^2) of 0.995 or better were obtained for thioTEPA and TEPA. The linear relationships were evaluated by least square regression analysis and by the LOF test. Performing the LOF test, α values of >0.05 indicate a linear relationship. Concentrations were back calculated from the peak areas and the relative concentration residuals (RCRs) were calculated from Eq. (4) [23]:

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

where RC = interpolated concentration, and NC = nominal concentration.

All RCR values were less than 10% and thus acceptable [22].

Calibration curves measured by GC–MS were determined in the concentration range of 50–25 000 ng/ml thioTEPA and TEPA in plasma or urine. A good linear relationship was found for TEPA in plasma and urine in the range of 100–25 000 ng/ml. A weighting factor of $1/x^2$ was applied, resulting in an r^2 of 0.993 and RCR of less than 15%. For the GC–MS analysis of thioTEPA in plasma a second order polynomial function was found to be appropriate to describe the concentration–detection response relation in the range of 500–25 000 ng/ml.

Therefore we selected the linear GC–TI-NPD assay for further analysis of clinical samples.

3.6. Accuracy and precision

In Tables 1 and 2 data of the assay performance are presented for thioTEPA and TEPA in plasma and urine, respectively. The accuracy, within- and between-run precision were within the acceptable criteria of $\leq$15% [22] for both thioTEPA and TEPA.

In Table 3 the accuracy and precision of the two dilution steps are shown. Values were always less than 10% and therefore samples with thioTEPA or

Table 1
Accuracy and precision of thioTEPA and TEPA analysis in plasma, using GC–TI-NPD

Nominal concentration (ng/ml)	Accuracy (%)		Within-run precision[a] (%)		Between-run precision[a] (%)	
	ThioTEPA	TEPA	ThioTEPA	TEPA	ThioTEPA	TEPA
10	2.6	−8.8	10.2	9.4	5.9	10.8
50	4.7	−7.3	6.5	8.2	4.3	8.2[b]
5000	3.0	−0.7	7.2	4.5	4.5	2.4

[a] Mean of three experiments, each done in quintuple.
[b] No statistical significant additional dispersion observed due to analysis between different runs.

Table 2
Accuracy and precision of thioTEPA and TEPA analysis in urine, using GC–TI-NPD

Nominal concentration (ng/ml)	Accuracy (%)		Within-run precision[a] (%)		Between-run precision[a] (%)	
	ThioTEPA	TEPA	ThioTEPA	TEPA	ThioTEPA	TEPA
10	2.0	−3.1	2.3	3.8	1.7	9.3
50	−1.0	−5.1	2.9	3.4	6.0	6.6
5000	−2.3	−2.7	2.8	4.5	3.2	6.7

[a] Mean of three experiments, each done in quintuple.

TEPA concentrations of more than 1 μg/ml can be diluted 10- or 100-fold.

3.7. Specificity and selectivity

No endogenous components interfered with the determination of thioTEPA, TEPA or diphenylamine, which was shown after the analysis of plasma and urine of six different individuals. Also cyclophosphamide and the co-medicated drugs ciprofloxacin, itraconazol, ranitidine, granisetron, dexamethason and lorazepam gave no interference with the determination of thioTEPA, TEPA and diphenylamine.

3.8. Sensitivity

The LODs for thioTEPA in plasma and urine were 1.5 ng/ml and 2.5 ng/ml, respectively. A LOD of 2.5 ng/ml in plasma and 5 ng/ml in urine was found for TEPA. Other reported assays using SPE [21,24] or chloroform with sodium chloride [16] to extract thioTEPA and TEPA possess comparable LODs, however require more biological specimens. Slightly more sensitive assays have been described, but different extraction methods were used for thioTEPA and TEPA [13] or only thioTEPA was determined [19].

Table 3
Accuracy and precision of diluted plasma samples spiked with thioTEPA and TEPA

Nominal concentration (ng/ml)	Accuracy (%)		Within-run precision[a] (%)		Between-run precision[a] (%)	
	ThioTEPA	TEPA	ThioTEPA	TEPA	ThioTEPA	TEPA
50	5.1	−4.0	7.2	4.9	–	3.9
500	3.0	−6.3	5.7	6.2	4.3	4.9

[a] Mean of three experiments, each done in quintuple.

The LOD of thioTEPA with the GC–MS method was 50 ng/ml in both plasma and urine. For TEPA a LOD of 100 ng/ml in plasma and 50 ng/ml in urine was measured. These results show that GC equipped with TI-NPD is more sensitive than GC–MS under our tested conditions.

3.9. Stability

Plasma and urine extracts of thioTEPA were stable at ambient room temperature in the autosampler over a period of 24 h. No decrease in concentration was observed for the urine extracts of TEPA, whereas the plasma extracts of TEPA gave a decrease of 12%. Therefore plasma extracts were kept in the autosampler for not more than 14 h. No significant decrease in concentration of TEPA in plasma and urine is seen during two freeze–thaw cycles (Table 4). Concentration dependable decrease of TEPA in plasma and urine was found when kept at 4°C or −20°C during a period of seven weeks. The percentage TEPA remaining after seven weeks is summarized in Table 5. Stability of TEPA in plasma and urine proved to be satisfactory for at least 10 weeks, when kept at −80°C. The plasma and urine concentrations was about 105% (±7.8) and 105% (±3.4) of the initial concentration, respectively.

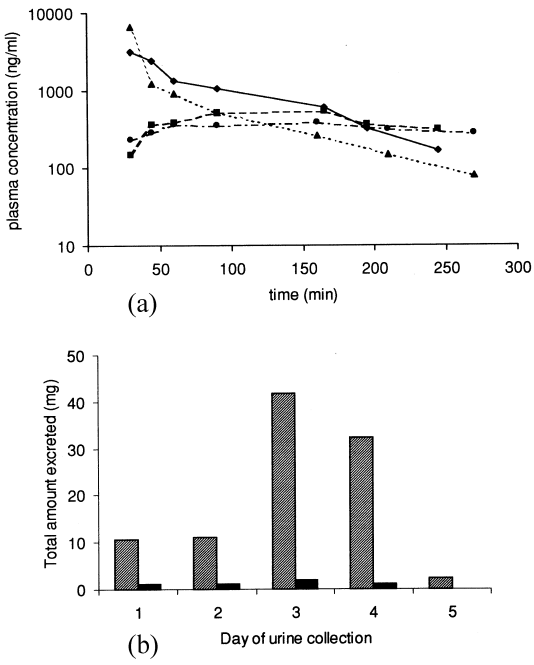

Fig. 4. (a) Mean plasma concentrations of thioTEPA and TEPA determined in two patients treated with 40 mg/m^2 (–▲– thioTEPA, –•– TEPA) or 60 mg/m^2 (–♦– thioTEPA, –■– TEPA). (b) Total amount of excreted thioTEPA and TEPA in urine of a patient treated with 40 mg/m^2 (thatched bars TEPA, black bars thioTEPA).

Table 4
Stability of TEPA in plasma and urine during two freeze–thaw cycles

Cycle	Plasma concentration (ng/ml)[a]			Urine concentration (ng/ml)[a]		
	10	50	500	100	500	5000
1	107±11.1	112±10.1	102±4.2	103±3.7	89±2.8	99±6.4
2	114±11.4	103±6.7	92±5.5	89±8.0	91±3.9	98±4.4

Percentage of initial concentration.
[a] Mean (±S.D.) of three experiments, each done in quintuple.

Table 5
Stability of TEPA in plasma and urine at 4°C and −20°C

Temperature	Plasma concentration (ng/ml)[a]			Urine concentration (ng/ml)[a]		
	10	50	500	100	500	5000
4°C	62±15		91±10	24±16	16±11	15±3.4
−20°C	61±12	85±10	90±3.2	30±6.4	48±4.3	68±1.0

Mean (±S.D.) percentage of the initial concentration.
[a] Mean (±S.D.) of three experiments, each done in quintuple.

3.10. Pharmacokinetic case study

In Fig. 4a the thioTEPA and TEPA plasma concentration–time profiles of two patients are depicted. The plasma concentration of thioTEPA decreases biexponentially, comparable with literature data [9,15]. As can be seen in Fig. 4a, still a substantial amount of TEPA (250–450 ng/ml) is present before the next thioTEPA infusion. The total amount thioTEPA and TEPA excreted in urine during each day of thioTEPA administration is shown in Fig. 4b. At day 5, the day after the last thioTEPA administration, some TEPA (2.3 mg) is still excreted.

4. Conclusions

The described GC–TI-NPD method combined with a liquid–liquid sample pre-treatment procedure is suitable for determination of thioTEPA and its metabolite TEPA in plasma or urine of patients treated with thioTEPA. With this assay thioTEPA and TEPA can be analysed in a single run after a single LLE of the sample. The sensitivity of GC–TI-NPD is a factor of 10–20 higher than GC–MS. The presented validated GC method can be used for pharmacokinetic clinical studies with thioTEPA.

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