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Liquid chromatographic–mass spectrometric determination of the novel, recently identified thioTEPA metabolite, thioTEPA-mercapturate, in urine

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Abstract

An assay for the quantitative determination of the mercapturic acid conjugate of *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA-mercapturate) in human urine has been developed. ThioTEPA-mercapturate, a recently identified metabolite of the alkylating anticancer agent thioTEPA, was analyzed using LC–MS and with direct sample injection. Sulphadiazine was used as internal standard. Linearity was accomplished in the therapeutic relevant range of 1–25 μg/ml; recovery was 84% and both accuracy and precision were less than 20% for the lower limit of quantification (1.0 μg/ml) and less than 10% for the other concentration levels. The stability of thioTEPA-mercapturate proved to be satisfactory over a period of 2 months, when kept at −80°C. ThioTEPA-mercapturate urine 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; ThioTEPA-mercapturate

1. Introduction

The chemotherapeutic alkylating drug *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA) has been applied in cancer therapy now for more than 40 years [1]. ThioTEPA possesses a broad spectrum of anti-tumor activity and is recently being employed in high-dose combination regimens for breast cancer, ovarian cancer and other solid tumors [2]. The first reported metabolite of thioTEPA is *N*,*N*′,*N*″-triethylenephosphoramide (TEPA) which is formed in the liver after oxidative desulfuration [3]. TEPA rapidly appears in plasma after thioTEPA infusion and has a half-life two to seven times longer than thioTEPA [4,5]. In a recent study a new metabolite of thioTEPA with alkylating capacity was identified as the mercapturic acid conjugate of thioTEPA (thioTEPA-mercapturate, Fig. 1) [6]. Mercapturate metabolites are conjugates of *N*-acetylcysteine and are the end-products of metabolic processes to detoxify potentially harmful electrophilic compounds [7]. The clinical importance of thioTEPA-mercapturate is not known yet. In order to get more quantitative insight into this new identified metabolic conver-

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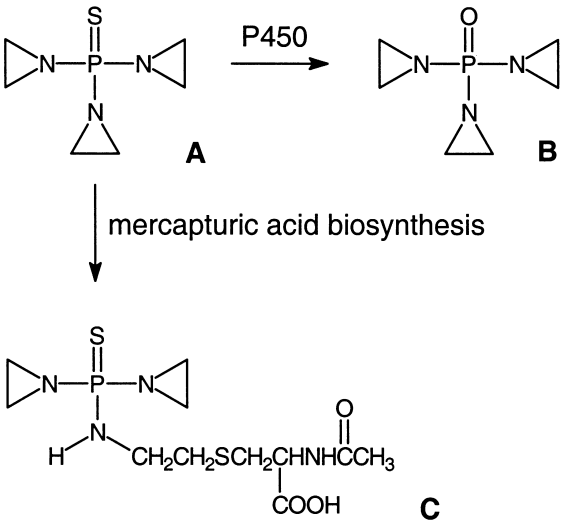

Fig. 1. Biotransformation of thioTEPA (A) to TEPA (B) and thioTEPA-mercapturate (C).

sion of thioTEPA an assay was developed and validated in which thioTEPA-mercapturate is analyzed in urine and which can be applied in clinical studies.

2. Experimental

2.1. Chemicals

ThioTEPA was obtained from Cyanamid Benelux (Etten-Leur, The Netherlands). *N*-Acetylcysteine was obtained from Sigma (St. Louis, MO, USA). Sulphadiazine, used as internal standard, originated from OPG (Utrecht, The Netherlands). All other chemicals used were of analytical grade unless otherwise specified.

2.2. Synthesis, isolation and identification of thioTEPA-mercapturate

A solution containing 200 μl 5 mg/ml thioTEPA, 40 μl 1 *M N*-acetylcysteine solution and 3.76 ml 28 m*M* sodium carbonate buffer, pH 11, was incubated for 15 min at 90°C. The solution was evaporated under a stream of nitrogen at room temperature to dryness. The residue was reconstituted in 500 μl water. For isolation four times a volume of 100 μl was injected into an HPLC system with UV detection operating at 205 nm. Separation was performed on a Lichrospher® 100 RP-18 (5 μm) column (125×4 mm, Merck, Darmstadt, Germany). Water was used as mobile phase and was delivered at a flow-rate of 1 ml/min. The peak eluting after 8 min was collected after each injection and the eluate was evaporated to complete dryness. The yield of thioTEPA-mercapturate was determined by weighing.

The identity and purity of the reaction product was established by fast atom bombardment-tandem mass spectrometry (FAB-MS–MS) and LC–MS.

2.3. Instrumentation

2.3.1. LC–MS

Separation was performed on a LiChrospher® 100 RP-18 (5 μm) column (125×4 mm, Merck). A mobile phase of 17.5% (v/v) acetonitrile in a 10 m*M* ammonium acetate buffer pH 4.8 was delivered at a flow-rate of 0.2 ml/min using a LC-10AD pump (Shimadzu, Tokyo, Japan). MS measurements were performed on a VG Platform, equipped with an electrospray ionization (ESI) source operating in the positive ion mode (Fisons Instruments, Beverly, MA, USA). Nitrogen was used as drying gas and as nebulizing gas at flow-rates of 400 and 15 l/h, respectively. The source temperature was set at 120°C and the cone voltage at 30 V. For thioTEPA-mercapturate and sulphadiazine, used as internal standard, ranges of m/z 350–360 and m/z 255–265 were scanned, respectively.

2.3.2. FAB-MS–MS

FAB-MS–MS measurements were carried out with a JMS-SX/SX102A tandem mass spectrometer (Jeol, Tokyo, Japan) operating at 10 kV and equipped with a Jeol FAB gun set at 10 mA emission current and producing a beam of 6 keV Xe atoms. Mass spectra were obtained by scanning MS-1, while product-ion spectra of the selected precursor ions were acquired by scanning MS-2 in the linked B/E scan mode using a collision cell in the third field free region of the instrument with air as the collision gas. The pressure of the collision gas was adjusted to obtain a 50% intensity of the main beam.

2.4. Calibration

Drug free urine was spiked with thioTEPA-mercapturate to yield the appropriate analyte concentrations. To 90 μl of these samples an aliquot of a 10 μl 100 μg/ml sulphadiazine solution was added and 10 μl was injected.

2.5. Validation

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

2.6. Specificity and selectivity

Urine samples of six different healthy volunteers were analyzed to check if endogenous components interfered with thioTEPA-mercapturate analysis.

2.7. Linearity

2.7.1. Lack of fit-test

The calibration curve ranged from 1 to 25 μg/ml 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) [8] 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.

2.7.2. Translational bias and rotational bias

To test if the method is subjected to translational or rotational bias the Student's *t*-test was applied [9] 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):

$$t_a = a/\mathrm{se}(a) \quad \text{and} \quad t_b = (b-1)/\mathrm{se}(b) \quad (3)$$

in which se 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.

2.7.3. 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.

2.8. Accuracy and precision

Quality control samples containing 1, 5, 17.5 and 25 μg/ml thioTEPA-mercapturate 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 analysis of variance (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.

2.9. Recovery

The recovery of thioTEPA-mercapturate was calculated by dividing the slopes of the calibration curves of thioTEPA-mercapturate in urine and in water, times 100%.

2.10. Stability

The stability of thioTEPA-mercapturate has been studied at concentrations of 1, 5, 17.5 and 25 μg/ml in urine over a period of 24 h at 22 and 4°C and 2 months at −80°C. The stability during two freeze–thaw cycles was studied at concentrations of 1, 5, 17.5 and 25 μg/ml in urine. For all stability studies five replicates were analyzed for each concentration level.

2.11. Pharmacokinetic case study

The described method was used to analyze thioTEPA-mercapturate urine concentrations of two patients treated with thioTEPA. The patients received thioTEPA twice daily 40 mg/m^2 in combination with cyclophosphamide 1000 mg/m^2 and carboplatin 265 mg/m^2 (tCTC regimen [10]). 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 thioTEPA-mercapturate

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 drug administration. Large volumes of urine were concentrated using solid-phase extraction (SPE) and fractionated with HPLC. Each fraction collected was assessed for alkylating activity using *p*-nitrobenzylpyridine as reagent. The fractions with alkylating activity were subjected to FAB-MS–MS and revealed the presence of a formally unknown metabolite. The molecular mass of this new metabolite found was 352 Da and equals the mass of thioTEPA-mercapturate. ThioTEPA-mercapturate could not be obtained as reference and was, therefore, synthesized in our laboratory as follows.

ThioTEPA was incubated with *N*-acetylcysteine in phosphate buffer. This resulted in a peak eluting after 8 min. The pH and temperature were varied to further optimise the formation of thioTEPA-mercapturate. Increased temperature and higher pH values resulted finally in a higher yield (with factor 2.5) of thioTEPA-mercapturate. The product was isolated with HPLC and its mass was determined with LC–MS, which gave a protonated molecular ion $[M+H]^+$ at m/z 353. FAB-MS–MS measurements resulted in a spectrum with two major fragment ions at m/z 310 and 267 (Fig. 2A). The ion at m/z 310 corresponds with loss of an aziridine molecule, also observed in the fragmentation pattern of thioTEPA. Loss of a second aziridine molecule results in the ion at m/z 267. In Fig. 2B the FAB-MS–MS spectrum of thioTEPA-mercapturate isolated from patient urine is shown, and which is identical to the synthesized product (Fig. 2A).

3.2. Analysis

GC analysis, as described previously for thioTEPA and TEPA [11], of a thioTEPA-mercapturate solution in methanol resulted in a broad peak at 8 min. Retention times were not reproducible and thioTEPA was formed due to thermal instability of thioTEPA-mercapturate. Reduction of the injector temperature or on-column injection resulted in even broader peaks which can be explained by the polar character of the analyte. HPLC was, therefore, then selected for thioTEPA-mercapturate analysis. The low specific absorption of thioTEPA-mercapturate made it necessary to detect at low wavelength. The mobile phase was buffered with ammonium acetate to protonate the free carboxylic acid present in thioTEPA-mercapturate. ThioTEPA-mercapturate gave a peak with a retention time of 5 min, using 10% (v/v) acetonitrile in 10 m*M* ammonium acetate, pH 4.8, as mobile phase delivered at 1 ml/min and UV detection at 205 nm. Liquid–liquid extraction (LLE) of a thioTEPA-mercapturate solution in water was tested with ethyl acetate, chloroform and mixtures of 1-propanol in chloroform which all resulted

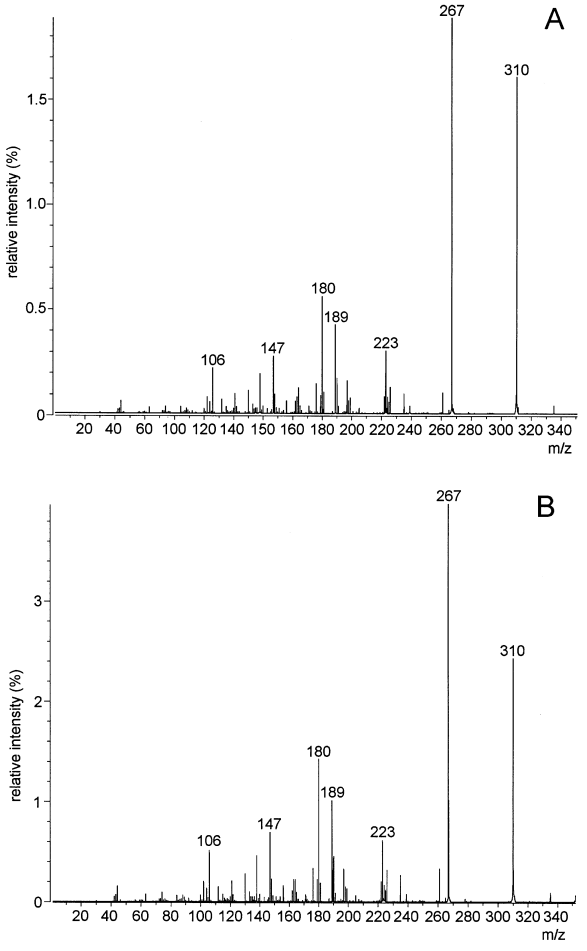

Fig. 2. FAB-MS–MS spectrum of thioTEPA-mercapturate, synthesized by incubation of thioTEPA with *N*-acetylcysteine for 30 min in 28 m*M* sodium carbonate buffer pH 11 at 95°C with precursor ion m/z 353 (A) and of the metabolite of thioTEPA isolated with HPLC after SPE extraction with precursor ion m/z 353 (B).

in recoveries of less than 10%. Reduction of pH to 4 or 5 improved recoveries to 50%, but were still not satisfactory. At lower pH values thioTEPA-mercapturate was subjected to degradation. With ion-exchange SPE of a thioTEPA-mercapturate solution in water the recovery improved to 80%. The eluate of ion-exchange SPE of thioTEPA-mercapturate in urine, however, contained a high amount of endogenous compounds, which interfered with the UV detection of thioTEPA-mercapturate at 205 nm. With the use of MS as detection no extraction procedure was necessary, whereas thioTEPA-mercapturate could be detected more selective. The recovery of thioTEPA-mercapturate was less than 100% (84±3.5%), due to the presence of endogenous compounds in the matrix which suppress the ionization. An internal standard was used to correct the variability of the ionization between different injections. Whereas a stable isotope of thioTEPA or acetylcysteine was not available, sulphadiazine appeared to be very useful as internal standard. The recovery of the internal standard was 95% (±2.8). For LC–MS measurements the flow was reduced to 0.2 ml/min and the amount of modifier was increased to 17.5%, resulting in retention times of 7.7 and 13.0 min for thioTEPA-mercapturate and sulphadiazine, respectively. With the described method thioTEPA and TEPA could not be detected simultaneously with thioTEPA-mercapturate, whereas thioTEPA and TEPA are subjected to degradation at the pH of the mobile phase [12,13]. Fig. 3 shows a chromatogram of a urine sample collected during 24 h on day 3 of the CTC regimen and in Fig. 4A and B the corresponding mass spectra of thioTEPA-mercapturate and the internal standard are shown.

3.3. Linearity

Calibration curves were constructed in the range of 1–25 μg/ml 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

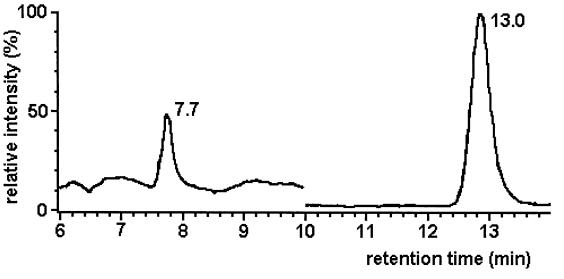

Fig. 3. LC–MS chromatogram of a patient urine sample collected on day 2 of the tCTC regimen. ThioTEPA-mercapturate concentration is 2.7 μg/ml. In the ranges of 6–10 and 10–14 min m/z 353 and 251 were selected, respectively. Peaks corresponding to retention times of 7.7 and 13.0 min are thioTEPA-mercapturate and sulphadiazine, respectively.

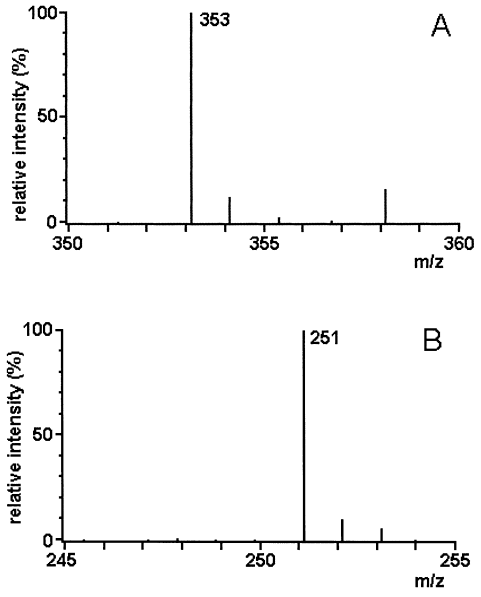

Fig. 4. Mass spectrum of thioTEPA-mercapturate in a patient urine sample spiked with sulphadiazine. The protonated molecular ions $[M+H]^+$ at m/z 353 (A) and m/z 251 (B) are derived of thioTEPA-mercapturate and sulphadiazine, respectively.

value, so $\alpha \approx 0$ and $\beta \approx 1$, indicating that there is no translational and 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 [14].

3.4. Accuracy and precision

In Table 1 data of the assay performance are presented for thioTEPA-mercapturate in urine. The accuracy, within-run and between-run precision were within the acceptable criteria of $\leq 15\%$ [14].

Table 1
Accuracy and precision of thioTEPA-mercapturate in urine

Nominal concentration (μg/ml)	Accuracy (%)[a]	Within-run precision (%)[a]	Between-run precision (%)[a]
1	−3.6	4.4	16.6
5	6.5	4.7	1.1
17.5	−2.0	5.3	7.8
25	−4.6	8.9	–[b]

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

3.5. Specificity and selectivity

No endogenous components interfered with the determination of thioTEPA-mercapturate, which was shown after the analysis of blank urine of six different individuals.

3.6. Sensitivity

The limit of detection (LOD) for thioTEPA-mercapturate in urine ranged between 0.1 and 0.5 μg/ml, and the LLQ at which the concentration can be determined with an accuracy of less than 20% for thioTEPA-mercapturate in urine was 1 μg/ml.

3.7. Stability

No significant decrease in concentration of thioTEPA-mercapturate in urine was observed at −80, 4 and 22°C (Table 2). The stability of thioTEPA-mercapturate proved to be satisfactory during two freeze–thaw cycles, the concentration remained about 102% (±10) of the initial concentration.

3.8. Pharmacokinetic case study

The total amount of thioTEPA, TEPA and thioTEPA-mercapturate excreted on each day of urine collection of two patients is depicted in Fig. 5. The amount of thioTEPA and TEPA has been measured with GC as previously described [11]. Whereas TEPA was assumed to be the main metabolite of thioTEPA, the excreted amount of thioTEPA-mercapturate is higher on each day of drug administration. At day 5, the day after the last thioTEPA administration, a substantial amount of thioTEPA-

Table 2
Stability of thioTEPA-mercapturate in urine at 4 and 22°C during 24 h and at −80°C during 2 months, calculated as percentage of the initial concentration

Temperature	Urine concentrations (μg/ml)			
	1.0	5.0	17.5	25.0
4°C	85±11	98±14	108±12	102±6.9
22°C	88±5.7	93±9.3	103±10	87±7.3
−80°C	94±6.5	104±6.5	95±3.2	89±12

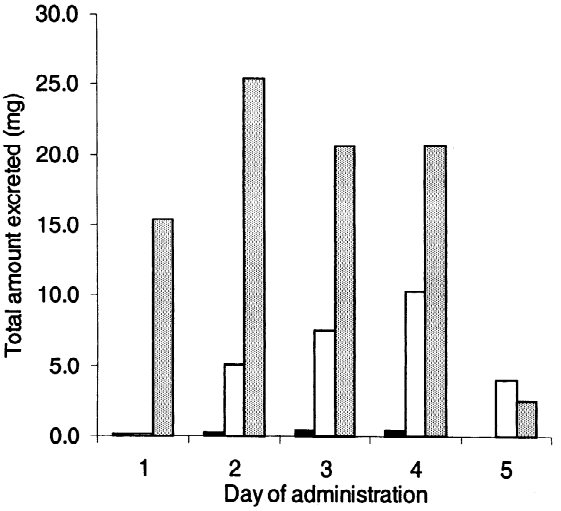

Fig. 5. Total amount of excreted thioTEPA (black bars), TEPA (white bars) and thioTEPA-mercapturate (dotted bars) in urine of two patients treated with 40 mg/m^2 thioTEPA, two times per day, on each day of urine collection.

mercapturate (2.6 mg) and TEPA (4.1 mg) is still excreted. The total amount excreted thioTEPA and metabolites, calculated as thioTEPA equivalents, was 6.2–16.4% of the administered dose. Considering the structural resemblance of TEPA with thioTEPA, the presence of TEPA-mercapturate in urine was investigated, but could not be detected.

4. Conclusions

The described LC–MS method is suitable for determination of thioTEPA-mercapturate in urine of patients treated with thioTEPA and can be used for pharmacokinetic clinical studies.

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