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Stability of thioTEPA and its metabolites, TEPA, monochloroTEPA and thioTEPA-mercapturate, in plasma and urine

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Abstract

The degradation of *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA) and its metabolites *N*,*N*′,*N*″-triethylenephosphoramide (TEPA), *N*,*N*′-diethylene,*N*″-2-chloroethylphosphoramide (monochloroTEPA) and thioTEPA-mercapturate in plasma and urine has been investigated. ThioTEPA, TEPA and monochloroTEPA were analyzed using a gas chromatographic (GC) system with selective nitrogen/phosphorous detection; thioTEPA-mercapturate was analyzed on a liquid chromatography-mass spectrometric (LC-MS) system. The influences of pH and temperature on the stability of thioTEPA and its metabolites were studied. An increase in degradation rate was observed with decreasing pH as measured for all studied metabolites. In urine the rate of degradation at 37°C was approximately 2.5 ± 1 times higher than at 22°C. At 37°C thioTEPA and TEPA were more stable in plasma than in urine, with half lives ranging from 9–20 h for urine and 13–34 h for plasma at pH 6. Mono- and dichloro derivatives of thioTEPA were formed in urine and the monochloro derivative was found in plasma. Degradation of TEPA in plasma and urine resulted in the formation of monochloroTEPA. During the degradation of TEPA in plasma also the methoxy derivative of TEPA was formed as a consequence of the applied procedure. The monochloro derivative of thioTEPA-mercapturate was formed in urine, whereas for monochloroTEPA no degradation products could be detected.

Keywords: ThioTEPA; TEPA; MonochloroTEPA; ThioTEPA-mercapturate; Stability in plasma and urine

1. Introduction

N,*N*′,*N*″-Triethylenethiophosphoramide (thioTEPA) is an alkylating agent that has been applied in cancer therapy for more than 40 years

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(Sykes et al., 1953). Nowadays thioTEPA is being employed mainly in high dose combination regimens for breast cancer, ovarian cancer and other solid tumors, because of its broad spectrum of antitumor activity and manageable toxicities (Vaughan et al., 1994; Van der Wall et al., 1995; Rodenhuis et al., 1996). ThioTEPA is mainly metabolized into two metabolites, *N*,*N*′,*N*″-triethylenephosphoramide (TEPA) and thioTEPA-mercapturate (Fig. 1). TEPA is formed after oxidative desulfuration of thioTEPA in the liver, catalyzed by cytochrome P450 (Hagen et al., 1991; Chang et al., 1995). ThioTEPA-mercapturate is likely the result of glutathione conjugation, after which the derivative loses two amino acid residues to yield the mercapturic acid conjugate (Chasseaud, 1976; Van Maanen et al., 1999c). *N*,*N*′-Diethylene,*N*″-2-chloroethylphosphoramide (monochloroTEPA; Fig. 1) was also found in urine of patients treated with thioTEPA but not in plasma (Van Maanen et al., 1999c). Degradation studies of thioTEPA in aqueous solutions (Benckhuijsen, 1968; Zon et al., 1976; Cohen, et al., 1984; Pyatigorskaya et al., 1987; Van Maanen et al., 1999b) have been performed. These studies showed the conversion of thioTEPA to chloro derivatives in acidic media and in the presence of chloride. Degradation of TEPA in aqueous solutions in the presence of sodium chloride resulted also in the formation of chloro derivatives (Van Maanen et al., 2000). No data, however, are available on the stabilities of TEPA, monochloroTEPA and thioTEPA-mercapturate in urine and plasma. Information about thioTEPA stability in biological fluids is also scarce (Cohen et al., 1984). In order to gain more insight into the chemical reactivity of thioTEPA and its metabolites in plasma and urine as well on the effects of acidity and temperature, we investigated the stability of thioTEPA and all its currently known metabolites (TEPA, monochloroTEPA, thioTEPA-mercapturate). Information about these stabilities are crucial for the bioanalysis of thioTEPA and metabolites.

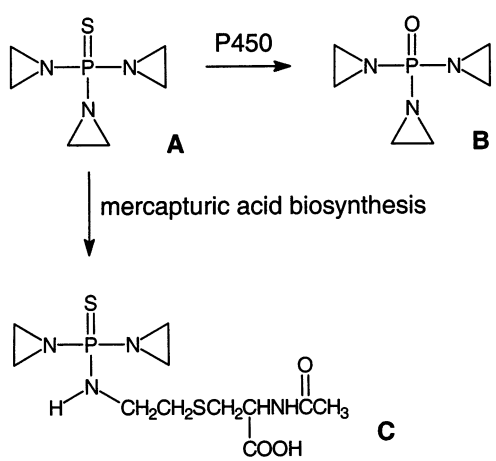


Fig. 1. Biotransformation of thioTEPA (A) to TEPA (B) and thioTEPA-mercapturate (C).

2. Materials and methods

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 (Van Maanen et al., 1998). Sulphadiazine and diphenylamine, used as internal standards, originated from OPG (Utrecht, The Netherlands) and Baker Analysed® Reagent (Deventer, The Netherlands), respectively. ThioTEPA-mercapturate and monochloroTEPA were synthesized as previously described (Van Maanen et al., 1999c). All other chemicals used were of analytical grade.

2.2. Analysis

2.2.1. ThioTEPA and TEPA

ThioTEPA and TEPA were dissolved to a concentration of 100 μg/ml in urine or plasma that was adjusted to specific pH values between 4 and 7 with acetic acid or sodium hydroxide. Samples were incubated at 22 and 37°C and at fixed time intervals an aliquot of 100 μl was withdrawn, 10 μl 1 M sodium hydroxide was added and the sample was stored at −80°C; the samples were analyzed within two weeks. Analysis and structure identification was done with gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS; Van Maanen et al., 1998).

Table 1
Half lives of the degradation of thioTEPA and TEPA in urine and plasma and of monochloroTEPA and thioTEPA-mercapturate in urine[a]

Medium	pH	Half lives (min)			
		ThioTEPA	TEPA	MonochloroTEPA	thioTEPA-mercapturate
Urine 22°C	4	30	17	31	71
	5	234	143	264	600
	6	2157	1248	7920	20340
Urine 37°C	4	16	5	12	26
	5	195	47	93	175
	6	1275	528	696	3528
Plasma 37°C	5	546	66	–[b]	–[b]
	6	2028	708	–	–
	7	7500	9120	–	–

[a] Stability tests were performed at 37°C for plasma and at 20 and 37°C for urine.
[b] As monochloroTEPA and thioTEPA-mercapturate were not found in plasma, their stability was not investigated in this medium.

2.2.2. MonochloroTEPA

MonochloroTEPA was dissolved to a concentration of 10 μg/ml in urine adjusted to pH 4, 5 or 6 with acetic acid or sodium hydroxide. Samples were incubated at 22 and 37°C and at fixed time intervals 100 μl samples were withdrawn, 10 μl 1 M sodium hydroxide was added and the samples were stored at −80°C until analysis. The extraction method used for the determination of thioTEPA and TEPA in urine as previously described (Van Maanen et al., 1998) was slightly modified for the analysis of monochloroTEPA. Thus, samples of 500 μl urine were transferred to polypropylene microcentrifuge tubes. 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 was vortexed for 15 s and centrifuged for 3 min at $1100 \times g$. The aqueous layer was discarded and the organic layer was transferred into a 0.7 ml amber glass vial. The organic solvent was evaporated under a nitrogen stream at room temperature to approximately 20 μl. Next, 30 μl methanol was added; 1 μl was injected into the chromatograph. Analysis and structure identification was performed with GC and GC-MS (Van Maanen et al., 1998).

2.2.3. ThioTEPA-mercapturate

The stability of thioTEPA-mercapturate was studied in urine adjusted to pH 4,5 or 6 with acetic acid or sodium hydroxide at a concentration of 20 μg/ml. Samples were incubated at 22 and 37°C and at fixed time intervals and aliquot of 90 μl was withdrawn. Following addition of 10 μl 1 M sodium hydroxide, the samples were stored at −80°C prior to analysis. Analysis and structure identification was performed using liquid chromatography-mass spectrometry (LC-MS; Van Maanen et al., 1999a).

3. Results

The stability of thioTEPA and its metabolites in urine and plasma strongly depends on the pH (Table 1), showing increased degradation at lower pH. The degradations followed pseudo-first order kinetics. Degradation rates in urine at 22°C are approximately 1.5–3 times lower than at 37°C. ThioTEPA and TEPA were far more stable in plasma than in urine at the same pH. TEPA proved to be the most reactive agent in urine and in plasma.

During the degradation of thioTEPA in urine two products were formed with GC retention

times of 6.3 min (D) and 10.3 min (E); thioTEPA (A) eluted at 4.1 min. Product D was also detected during the degradation in plasma. GC-MS analysis of thioTEPA (m/z 189) showed loss of aziridine (m/z 147), and aziridine and sulphur (m/z 115; Fig. 2a). The mass spectrum of product D showed a molecular ion at m/z 225 with an intense isotope peak at 227 (Fig. 2b). The ratio 3:1 for m/z 225 and 227 indicates the presence of a chlorine atom. The mass spectrum shows loss of chlorine (m/z 190), hydrogen chloride (m/z 189), aziridine (m/z 183), chloroethylamine (m/z 147), chloroethylamine and sulphur (m/z 115); m/z 42 originates from an aziridine group. For product E a molecular ion at m/z 261 with isotopes at m/z 263 and 265 at a ratio of 9:6:1, indicating the presence of two chlorine atoms. Loss of chlorine (m/z 226), hydrogen chloride (m/z 225) and chloroethylamine (m/z 183) was observed (Fig. 2c).

Degradation of TEPA (B) in urine resulted in the detection of one product with GC retention time of 5.2 min (F); TEPA eluted at 3.5 min. The

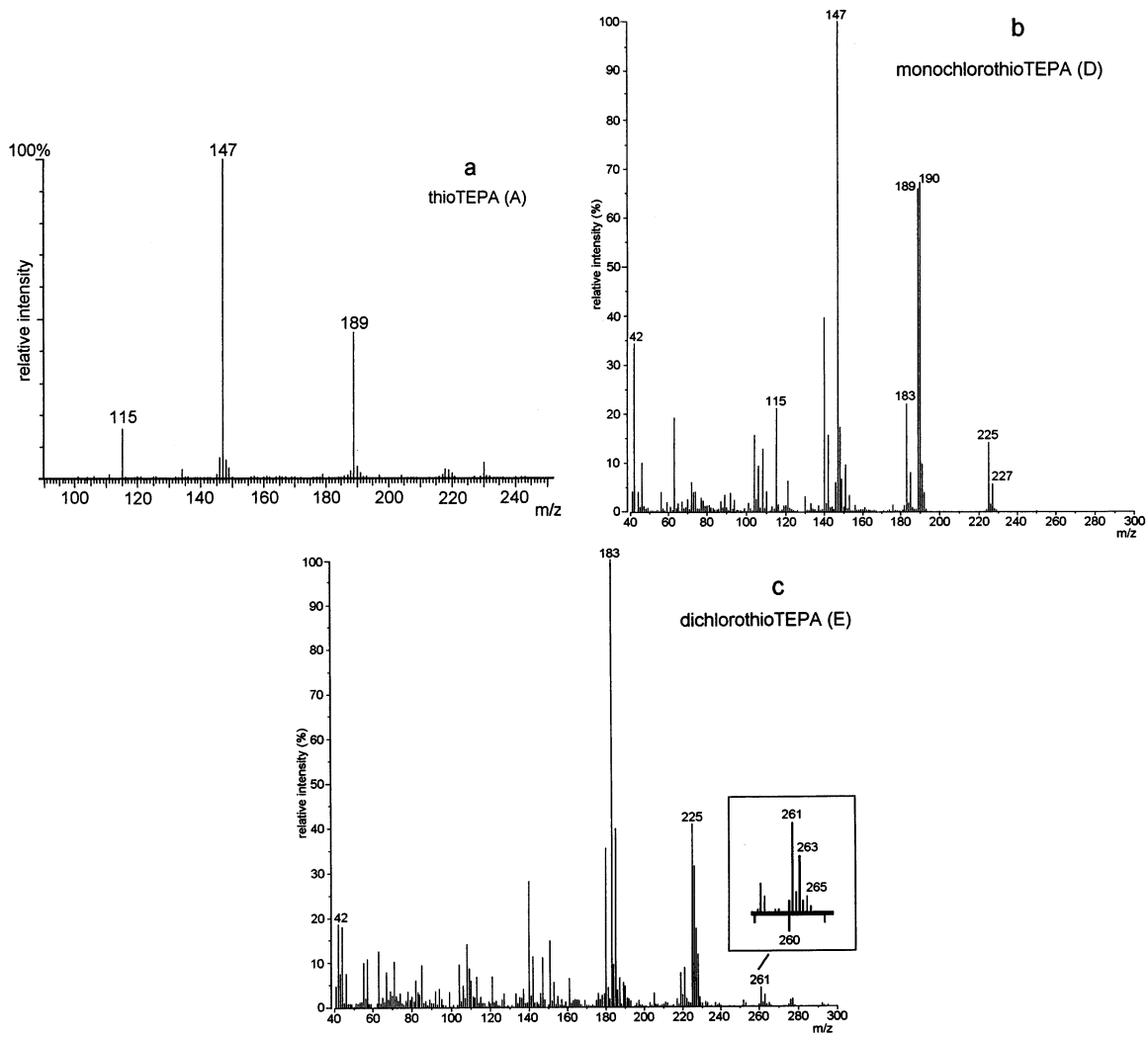


Fig. 2. Mass spectra of thioTEPA (product A, a) and its degradation products with t_r 6.3 min (D, b) and t_r 10.3 min (E, c); product D was formed in plasma, products D and E were formed in urine.

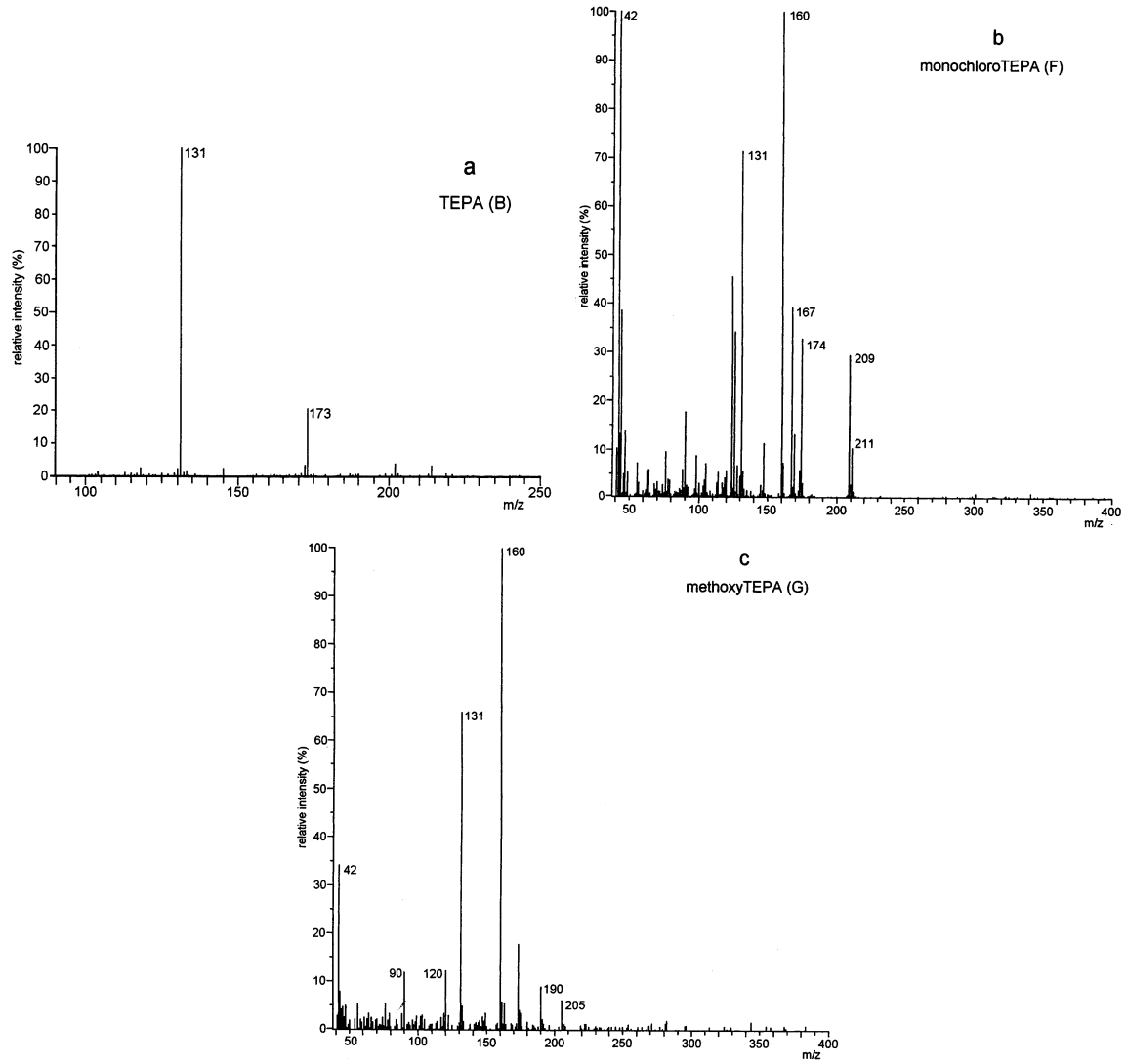


Fig. 3. Mass spectra of TEPA (B, a) and its degradation products with t_r 5.2 (F, b) and t_r 4.9 (G, c); product F was formed in plasma and urine, the product with t_r 4.9 was formed in plasma.

mass spectrum of TEPA (m/z 173) is depicted in Fig. 3a and shows loss of aziridine (m/z 131). The mass spectrum of product (F) showed a molecular ion at m/z 209 with an isotope at 211 (Fig. 3b). The ratio 3:1 for m/z 209 and 211 indicates the presence of one chlorine atom. The mass spectrum shows loss of chlorine (m/z 174), aziridine (m/z 167), chloromethyl (m/z 160), and chloroethylamine (m/z 131). The mass spectrum is in agreement with the assigned structure of monochloroTEPA. Only one product, with a retention time of 4.9 min, was detected after the degradation of TEPA in plasma. The mass spectrum (Fig. 3c, product G) revealed a molecular ion at m/z 205 and showed loss of methyl (m/z 190), methylmethoxy (m/z 160) and methoxyethylamine (m/z 131). This indicates for the formation of the methoxy derivative of TEPA (Fig. 5).

Degradation of thioTEPA-mercapturate (C) in urine resulted in the formation of one product,

which gave a molecular ion at m/z 389 as verified by LC-MS (H, data not shown). No detectable products were formed during the degradation of monochloroTEPA in urine.

4. Discussion

Data on the degradation of thioTEPA and its metabolites in plasma and urine are scarce. Cohen and co-workers studied the stability of thioTEPA in urine of which the pH was adjusted with HCl or NaOH (Cohen et al., 1984). In urine adjusted to pH 4.0 with HCl the monochloro and dichloro derivatives of thioTEPA were detected; no data on the stability of TEPA in urine were presented. The chemical degradation of thioTEPA and TEPA in aqueous solutions in the absence and presence of chloride ions, however, has been described in several studies (Mellet and Woods, 1960; Benckhuijsen, 1968; Maxwell et al., 1974; Zon et al., 1976; Pyatigorskaya et al., 1987; Van Maanen et al., 1999b, 2000).

A pH dependable degradation is seen for thioTEPA and its metabolites. TEPA appeared to be the most reactive agent, with half lives 2–3 times shorter than for thioTEPA. The reaction rate of thioTEPA in urine is in the same order of magnitude as reported by Cohen et al. (1984). The half lives of thioTEPA and TEPA in plasma at 37°C are over 2 days. This indicates that both analytes will also be stable at temperatures of 4 and 20°C, at which both thioTEPA and TEPA are most likely to be exposed to during analytical studies. Mass spectrometric analysis of the degradation products of thioTEPA revealed the formation of mono- (Fig. 2b) and dichloro derivative (Fig. 2c) of thioTEPA in urine and the monochloro derivative in plasma (Fig. 4). The formation of chloro derivatives of thioTEPA in urine was consistent with the products found by Cohen et al. (1984). The degradation of TEPA in plasma and urine also revealed the formation of chloro derivatives (Fig. 3 and Fig. 4). In our previous study monochloroTEPA was identified as a metabolite of thioTEPA (Van Maanen et al., 1999c). The results of this study show that monochloroTEPA can thus also be formed in vitro in urine. To preclude in vitro formation of monochloroTEPA, urine samples must be stored at -80°C immediately after voiding; under these conditions the analytes are stable for at least 10

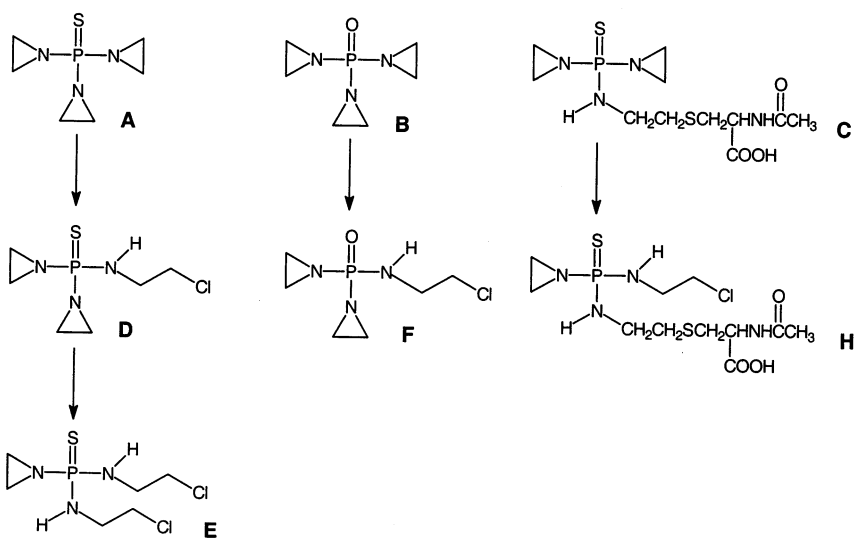
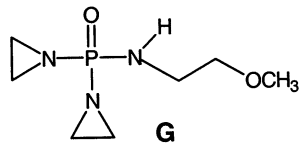


Fig. 4. Degradation scheme of thioTEPA (A), TEPA (B) and thioTEPA-mercapturate (C) in biological fluids. In plasma the monochloro derivatives of thioTEPA and TEPA (D and F, respectively) were formed. In urine the monochloro (D) and dichloro derivative (E) of thioTEPA, monochloroTEPA (F) and the monochloro adduct of thioTEPA-mercapturate (H) were formed.



G

Fig. 5. Methoxy derivative of TEPA (G) formed as consequence of the applied sample pretreatment.

weeks (Van Maanen et al., 1998). The product, detected during the degradation experiments with TEPA in plasma with a retention time of 4.9 min appeared to be the methoxy derivative of TEPA (Fig. 3c and Fig. 5), which is formed due to the applied method. To initiate the degradation, an aliquot of a TEPA solution in methanol is added to the buffer solution (100 μl methanol to 900 μl urine). Methanol, as a nucleophilic reagent gives a substitution reaction with TEPA. If a solution of TEPA in ethanol (10% v/v) is used, a product with a retention time of 11.3 min was formed. The mass spectrum of this product showed a molecular mass of m/z 219 and loss of ethyl (m/z 190), methylethoxy (m/z 160), ethoxyethylamine (m/z 131), and is consistent with the ethoxy derivative of TEPA (Van Maanen et al., 2000). The degradation products of thioTEPA and TEPA were also found during degradation studies in aqueous solutions (Zon et al., 1976; Van Maanen et al., 1999b, 2000). However, during the degradation of thioTEPA in aqueous solutions in the presence of sodium chloride (1 M) at pH < 7 also the trichloro derivative is formed (Van Maanen et al., 1999b). This product was not detected during the degradation of thioTEPA in urine. Degradation of TEPA in aqueous solutions showed the formation of the dichloro derivative of TEPA, which was also not formed in urine (Van Maanen et al., 2000). As can be seen in the degradation studies of thioTEPA and TEPA in aqueous solutions, the formation of the chloro derivatives depends on the concentration of the sodium chloride. The trichloro derivatives of thioTEPA and the dichloro derivative of TEPA emerge at sodium chloride concentrations in the incubation solution above 300 mM. In plasma the physiological chloride concentration is approximately 155 mM. This lower chloride concentration in plasma may explain the absence of the trichloro derivatives of thioTEPA and the dichloro derivative of TEPA during our stability studies in plasma. On the other hand thioTEPA and TEPA in urine are 0.5–2 times less stable in aqueous solutions in the presence of 1 M sodium chloride but at identical pHs (Van Maanen et al., 1999b, 2000). Consequently, thioTEPA and TEPA are also subjected to other degradation reactions in urine, of which, apparently the products could not be detected.

The mass of the product formed during the decomposition of thioTEPA-mercapturate in urine has a mass equal to the monochloro derivative of thioTEPA-mercapturate. The isotope at m/z 391 could not be observed, as the background signal gave an interfering ion at this m/z value. In addition to the degradation of TEPA, it would be expected that monochloroTEPA was converted to the dichloro derivative of TEPA, but such degradation products were not detected. In Fig. 4 the degradation scheme of thioTEPA, TEPA and thioTEPA-mercapturate in the tested biological fluids is depicted.

This study shows that thioTEPA and its metabolites are very reactive in urine at physiological pH. In plasma both thioTEPA and TEPA are stable with half lives of more than 2 days. To prevent degradation of these agents, collected samples must be stored at −80°C immediately after sampling, where they can be kept for 10 weeks without deterioration before analysis (Van Maanen et al., 1997, 1998).

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