A comprehensive understanding of thioTEPA metabolism in the mouse using UPLC–ESI-QTOFMS-based metabolomics

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ARTICLE INFO

ABSTRACT

ThioTEPA, an alkylating agent with anti-tumor activity, has been used as an effective anticancer drug since the 1950s. However, a complete understanding of how its alkylating activity relates to clinical efficacy has not been achieved, the total urinary excretion of thioTEPA and its metabolites is not resolved, and the mechanism of formation of the potentially toxic metabolites S-carboxymethylcysteine (SCMC) and thiodiglycolic acid (TDGA) remains unclear. In this study, the metabolism of thioTEPA in a mouse model was comprehensively investigated using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC–ESI-QTOFMS) based-metabolomics. The nine metabolites identified in mouse urine suggest that thioTEPA underwent ring-opening, N-dechloroethylation, and conjugation reactions in vivo. SCMC and TDGA, two downstream thioTEPA metabolites, were produced from thioTEPA from two novel metabolites 1,2,3-trichloroTEPA (VII) and dechloroethyltrichloroTEPA (VIII). SCMC and TDGA excretion were increased about 4-fold and 2-fold, respectively, in urine following the thioTEPA treatment. The main mouse metabolites of thioTEPA in vivo were TEPA (II), monochloroTEPA (III) and thioTEPA-mercapturate (IV). In addition, five thioTEPA metabolites were detected in serum and all shared similar disposition. Although thioTEPA has a unique chemical structure which is not maintained in the majority of its metabolites, metabolomic analysis of its biotransformation greatly contributed to the investigation of thioTEPA metabolism in vivo, and provides useful information to understand comprehensively the pharmacological activity and potential toxicity of thioTEPA in the clinic.

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1. Introduction

The alkylating agent, thioTEPA (N,N′,N″-triethylenethiophosphoramide), is used as an anti-cancer drug [1,2], and commonly employed for the treatment of breast, ovarian, and bladder cancer, and bone marrow ablation in combination regimens. ThioTEPA is believed to exert its pharmacological activity through the generation of DNA adducts [3,4]. It has been proposed that thioTEPA is a polyfunctional alkylating agent which can interact with DNA by two different pathways. One is the formation of interstand cross-links between thioTEPA and DNA molecules due to its alkylating activity [5,6]. Another suggestion is that thioTEPA is a prodrug for aziridine, which can result in the formation of guanine adducts and cause DNA chain scission [7].

To date, three metabolites TEPA, monochloroTEPA, and thioTEPA-mercapturate have been reported in vivo [8], and these also possess alkylating activity. ThioTEPA-mercapturate was generated from thioTEPA through the intermediate metabolites monoglutathionylthioTEPA and thioTEPA-cysteinate [8,9]. Previous studies have suggested that cytochrome P450s CYP3A4, 2B1, 2B6, and 2C11 were the primary metabolic enzymes involved in the conversion of thioTEPA to TEPA [10–12]. In addition, monochloroTEPA can be produced non-enzymatically from TEPA in vivo and in vitro, which is highly dependent on pH [13].

ThioTEPA pharmacokinetics remained largely unknown until the progression of gas chromatography in the 1980s. Previous clinical studies suggested that thioTEPA can be quickly eliminated from serum ($t_{1/2\beta}$ = 43–174 min), but TEPA persisted longer in the serum with a half-life of 3–21 h [14,15]. Human urinary excretion of unchanged thioTEPA, TEPA, monocholoroTEPA, and thioTEPA-mercapturate was 0.1–1.5%, 1.8–25.5%, 0.3–0.8% and 6.3–22.7% of

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the total administered thioTEPA, respectively [16]. In these studies, there were significant differences between the excreted total alkylating activity and the total quantitative excretion of thioTEPA and its metabolites [8,17,18], suggesting the presence of unknown metabolites of thioTEPA.

In the clinic, encephalopathy has been observed following treatment with high doses of thioTEPA [19–21]. It has been reported that S-carboxymethylcysteine (SCMC) and thiodiglycolic acid (TDGA) might be the possible toxic metabolites involved in ifosfamide-induced encephalopathy [22–24], and, interestingly, SCMC and TDGA have also been detected in human urine after administration of thioTEPA and at low levels in the urine of drug-free normal human volunteers [25,26]. However, how SCMC and TDGA are formed from thioTEPA remains unclear.

Metabolomics, the global unbiased analysis of the metabolites present in fluids, tissues or cells, is evolving as a useful tool in drug research and development [27–29]. Various analytical techniques [such as ultra-performance chromatography-linked time-of-flight mass spectrometry (UPLC–TOFMS), gas chromatography mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR)], and multivariate data analysis [such as principal components analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA)] have been applied in metabolomic-based drug metabolism studies. In terms of understanding drug metabolism using UPLC–ESI-QTOFMS-based metabolomics, there have been many successful applications, such as determining the metabolic maps of acetaminophen [30], melatonin [31], arecoline [32], and, recently, ifosfamide and cyclophosphamide [33].

UPLC–ESI-QTOFMS-based metabolomics was applied to understand better the metabolism of thioTEPA in vivo. The present study sheds new light on the metabolic pathways of thioTEPA in vivo, especially in the production of two important endogenous metabolites, SCMC and TDGA. The current study contributes to our understanding of the mechanisms underlying safety and efficacy of thioTEPA in the clinic.

2. Materials and methods

2.1. Chemicals and reagents

ThioTEPA, SCMC, TDGA, chloroacetaldehyde (CAA), 4-nitrobenzoic acid, debrisoquine, and NADPH were purchased from Sigma–Aldrich (St. Louis, MO). All solvents for UPLC–ESI-QTOFMS and triple quadrupole mass spectrometry and other chemicals were of the highest grade commercially available.

2.2. In vitro metabolism of thioTEPA in mouse liver microsomes

Livers from untreated 6- to 8-week-old age male C57BL/6 mice were homogenized, and microsomes were prepared as described previously [34]. Microsomal incubations were carried out in 50 mM Tris–HCl buffer solution, pH 7.4, containing 0.5 mg/ml microsomal protein, 2 mM $MgCl_2$, 20 μM thioTEPA, and 2 mM freshly prepared NADPH in a final volume of 200 μl. After 30 min incubation at 37 °C, the reaction was stopped using dry ice, 100 μl of the reaction mix was transferred to a new tube and acetonitrile (100 μl) directly added to precipitate protein. After centrifuging at 14,000 × g for 20 min, an 5 μl aliquot of the supernatant was injected into the UPLC–ESI-QTOFMS.

2.3. In vivo metabolism of thioTEPA in mice

Male C57BL/6 mice 6–8 weeks age were used for urine and serum metabolite profiling. Mice were housed four to five animals per cage and maintained under a standard 12 h light, 12 h dark cycle with water and chow provided ad libitum. Handling was in accordance with an animal study protocol approved by the National Cancer Institute Animal Care and Use Committee. ThioTEPA dissolved in saline solution was administrated by intraperitoneal injection (i.p.) at the dose of 50 mg/kg (approximately 1 mg of thioTEPA). Control mice were treated with saline alone. Urine samples were collected from mice housed in glass metabolic cages (Metabowls, Jencons Scientific USA, Bridgeville, PA). Blood was collected in BD microtainer® serum separator tubes (Franklin Lakes, NJ) by retroorbital bleeding after thioTEPA treatment. Serum was isolated following centrifugation at 8000 × g for 15 min. Ten mice were used for the identification of thioTEPA metabolites in urine and serum after treatment for 24 h and 1 h, respectively, including 5 control and 5 thioTEPA treatment mice. The urine samples for the excretion experiment were collected at 1, 4, 10, and 24 h following treatment with either thioTEPA or saline (controls). The blood samples for the pharmacokinetic study were collected at 0.08, 0.25, 0.5, 1, 4, 10, and 24 h after thioTEPA or control treatment. Four mice were sampled in each time point.

In addition, CAA, SCMC, and TDGA dissolved in saline solution were administrated by intraperitoneal injection (i.p.) at the dose of 2, 100, and 100 mg/kg (approximately 0.04, 2, and 2 mg drug), respectively. Control mice were treated with saline alone. Each treatment group included 4 mice. 24 h urine samples were collected in glass metabolic cages after treatment. Serum samples were also harvested from each group after being treated for 24 h. All the urine and serum samples were separately stored at −80 °C before analysis.

2.4. UPLC–ESI-QTOFMS identification of thioTEPA metabolites

Urine samples were prepared by mixing 20 μl urine with 180 μl 50% aqueous acetonitrile. Serum samples were prepared by mixing 10 μl serum with 190 μl 66% aqueous acetonitrile. After a centrifugation at 14,000 × g for 20 min, a 5 μl aliquot of the supernatants was injected into a Waters UPLC–ESI-QTOFMS system (Waters Corporation, Milford, MA). The chemical components in urine, serum, or from microsome incubations mix were separated using an Acquity C_{18} BEH UPLC column (Waters Corporation). The flow rate 0.5 ml/min was maintained in a 10 min run. Solvent A was composed of water and 0.1% formic acid and solvent B was composed of acetonitrile and 0.1% formic acid. The gradient was maintained at 100% A for 0.5 min, increased to 100% B over the next 7.5 min and returned to 100% A in last 2 min. Data were collected in both positive ion mode and negative ion mode on a Waters Q-TOF Premier mass spectrometer, which was operated in full-scan mode at 50–850 m/z. Nitrogen was used as both cone gas (50 l/h) and desolvation gas (600 l/h). Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. The capillary voltage and cone voltage were 3000 and 20 V, respectively. The structures of thioTEPA metabolites were elucidated by tandem MS fragmentography with collision energies ranging from 15 to 40 eV.

2.5. Data processing and multivariate data analysis (MDA)

Centroided and integrated mass spectrometric data were deconvoluted by MarkerLynx software (Waters Corporation). A multivariate data matrix containing sample identity, m/z, retention time, and abundance was generated through peak recognition and integration. MetaboLynx software (Waters Corporation) was also used to generate a table of ions that resulted from putative thioTEPA-derived metabolites, such as TEPA. The data matrix was analyzed by SIMCA-P + 12.0 software (Umetrics, Kinnelon, NJ). OPLS was conducted to identify the metabolites by comparing

thioTEPA-treated mice and control mice on the basis of the corresponding loading plots and contribution lists.

2.6. UPLC–ESI-QTOFMS quantitation of thioTEPA and its metabolites

QuanLynx software was applied to quantitate the amounts of thioTEPA and its metabolites in the urine and serum from their peak areas. The calibration curve of thioTEPA ($[M+H]^+$ = 190.057) was constructed from 0.19 μM to 100.0 μM using debrisoquine (1 μM; $[M+H]^+$ = 176.118) as the internal standard. The concentration of thioTEPA in urine and serum was calculated by the peak area ratio (analyte/debrisoquine). Because the standards were unavailable for thioTEPA metabolites, their relative intensity in urine and serum were estimated by comparing the peak areas from the total ion chromatogram.

2.7. Triple quadrupole mass spectrometry quantitation of SCMC and TDGA

Deproteinated urine samples from thioTEPA-treated mice were analyzed on an Applied Biosystems API 2000 ESI triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) using a Luna C_{18} column (Phenomenex, Torrance, CA, 50 mm × 4.6 mm i.d.). The mobile phase was 95% aqueous acetonitrile containing 0.1% formic acid. The flow rate was set at 0.2 ml/min. The mass spectrometer was operated in ESI negative ion mode. Nitrogen was used as the turbo ionspray and nebulizing gas. A voltage of 5 kV was applied to the sprayer needle, and turbo ionspray temperature was maintained at 350 °C. The calibration curves of SCMC

($178.0 \rightarrow 77.9$) and TDGA ($149.0 \rightarrow 92.7$) were constructed from 0.19 μM to 50.0 μM. 4-Nitrobenzoic acid ($165.9 \rightarrow 109.6$) was used as an internal standard. Absolute peak areas were used to calculate peak area ratios (analyte/4-nitrobenzoic acid).

2.8. Serum clinical chemistry

Serum amylase, glucose, alanine transaminase (ALT), alkaline phosphatase (ALP), and other physiological serum parameters were measured using the VetScan VS2 Comprehensive Diagnostic Profile (Abaxis, Inc., Union City, CA).

2.9. Statistic analysis

Half-life was calculated using WinNonlin (SCI, Apex, NC). Experimental values are expressed as mean ± S.D. Statistical analysis was performed using independent Student's t tests.

3. Results

3.1. Metabolites of thioTEPA formed by mouse liver microsomes

Ng and Waxman [10], using gas chromatography, reported only one thioTEPA metabolite formed from phenobarbital-induced rat hepatic microsomal incubations. In the present study, thioTEPA was incubated with non-induced mouse liver microsomes for 30 min and the deproteinated incubation was analyzed using UPLC–ESI-QTOFMS. MetaboLynx software (Waters Corp.) was used to generate a table of ions composed of theoretical thioTEPA
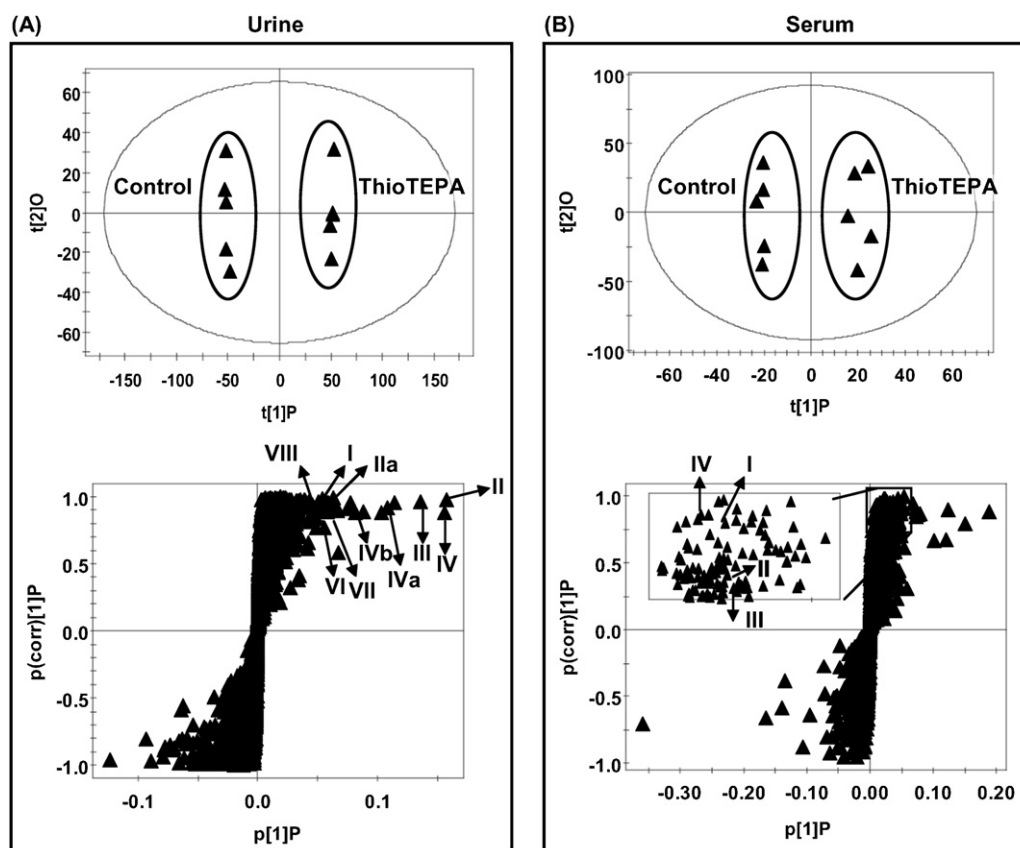

Fig. 1. Identification of thioTEPA metabolites in urine and serum using UPLC–ESI-QTOFMS-based metabolomics. (A) Scores plot of an OPLS model and OPLS loadings S-plot of urinary ions from control and thioTEPA-treated mice. Each point represents an individual mouse urine sample (top) and a urinary ion (bottom). ThioTEPA and its metabolites are labeled in the S-plot (I, II, IIa, III, IV, IVa, IVb, V, VI, VII, and VIII). (B) Scores plot of an OPLS model and OPLS loadings S-plot of serum ions from control and thioTEPA-treated mice. Each point represents an individual mouse serum sample (top) and a serum ion (bottom). ThioTEPA and its metabolites are labeled in the S-plot (I, II, III, and IV). The t[1] and t[2] correspond to principal components 1 and 2, respectively. The p(corr)[1]P values represent the interclass difference and p[1]P values represent the relative abundance of the ions. TDGA and SCMC were best detected in negative ion mode (ESI−) and therefore they are not shown in the OPLS loadings S-plot of positive ions (ESI+).
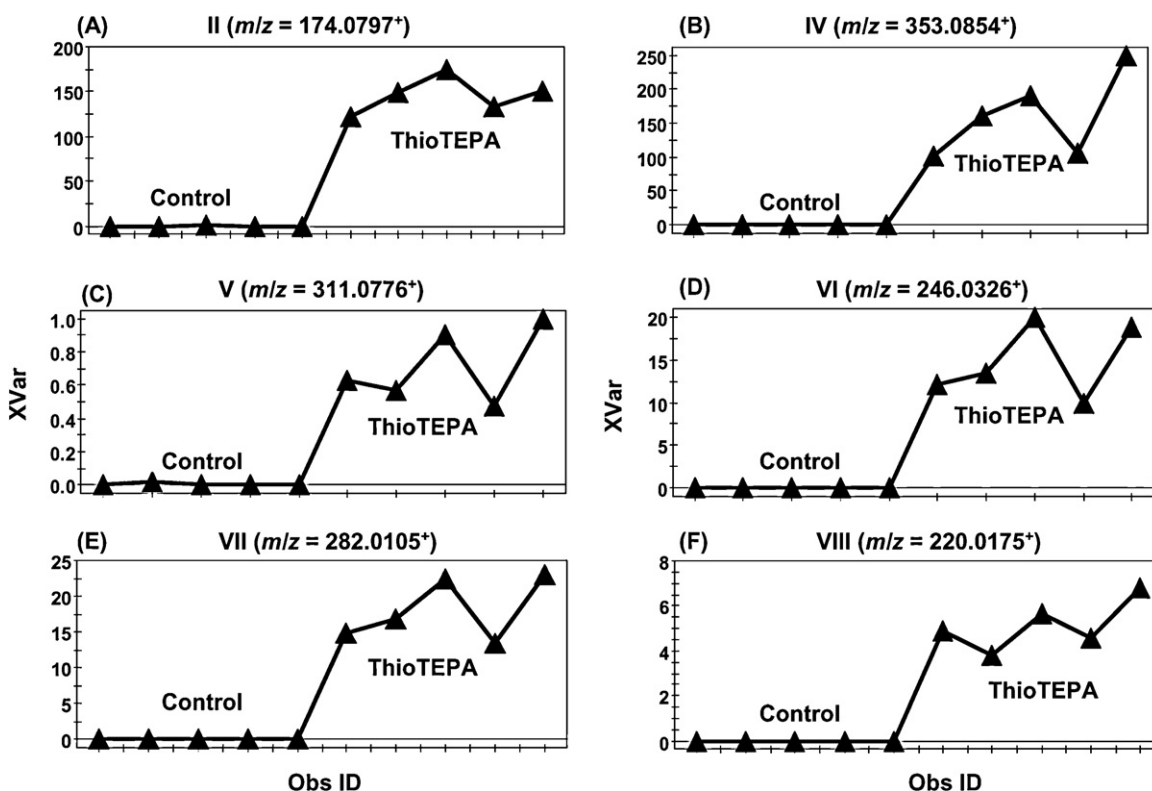
Fig. 2. Trend plots of ions from thioTEPA metabolites in control and thioTEPA-treated mouse urines 24 h post-dose. (A) Metabolite II with m/z value of 174.0797$^+$. (B) Metabolite IV with m/z value of 353.0854$^+$. (C) Metabolite V with m/z value of 311.0776$^+$. (D) Metabolite VI with m/z value of 246.0326$^+$. (E) Metabolite VII with m/z value of 282.0105$^+$. (F) Metabolite VIII with m/z value of 220.0175$^+$. Note the absence of putative drug metabolites in the control group. Metabolite codes correspond to those in Fig. 1. Obs ID represents the mouse urine sample and XVar values represent the relative abundance of the ions.

metabolites. Combined with the extracted MS spectrum from the total ion chromatogram of the control (without NADPH) and test (with NADPH) samples, TEPA ([M+H]$^+$ = 174.079) was the sole metabolite from thioTEPA generated by the in vitro system.

3.2. Identification and structural elucidation of thioTEPA metabolites in urine

Although just one thioTEPA metabolite was identified in vitro, two metabolites monochloroTEPA and thioTEPA-mercapturate were described in human urine [8]. Previous results suggested that thioTEPA can be transformed to other metabolites through non-cytochrome P450 pathways. To achieve a comprehensive description of urinary metabolites, metabolomic analysis was applied to screen for thioTEPA metabolites. As shown in the scores scatter plot (Fig. 1A), urine samples from the thioTEPA-treated group were significantly separated from the control group by the presence of thioTEPA and thioTEPA metabolites. The ions were identified as the putative thioTEPA metabolites based on the trend plots (Fig. 2A–F). The chemical compositions and structures were determined by accurate mass measurements and further elucidated by MS/MS fragmentography. The results revealed the presence of thioTEPA parent ion (I), TEPA (II), monochloroTEPA (III), thioTEPA-mercapturate (IV), the intermediate product thioTEPA-cysteinate (V), two new metabolites (VII–VIII), and two potential toxic metabolites SCMC (IX) and TDGA (X) in the thioTEPA-treated mouse urine sample (Table 1). The mass spectra are presented in Fig. 3A–F.

Metabolite VI (1,2-dichloroTEPA) was calculated as $C_6H_{14}Cl_2N_3OP$ based on the accurate mass measurement, m/z 246.0326$^+$. Comparison of the chemical composition with TEPA

Table 1
Identified thioTEPA metabolites in mouse urine.

| Symbol	RT (min)	Observed m/z	Formula	Mass error (ppm)	Identity	MS/MS fragments
I	3.60	190.0574	$C_6H_{12}N_3PS$ [H$^+$]	3.2	ThioTEPA	190; 147; 104
II	1.84	174.0797	$C_6H_{12}N_3OP$ [H$^+$]	0.6	TEPA	174; 131; 90
IIa	1.84	131.0380	$C_4H_7N_2OP$ [H$^+$]	4.6	TEPA fragment	
III	2.32	210.0587	$C_6H_{13}ClN_3OP$ [H$^+$]	11.4	MonochloroTEPA	210;167;126;112; 90
IV	3.95	353.0854	$C_{11}H_{21}N_4O_3PS_2$ [H$^+$]	−4.8	ThioTEPA-mercapturate	353; 310; 267; 223; 198; 156; 112
IVa	3.95	267.0031	$C_7H_{11}N_2O_3PS_2$ [H$^+$]	1.5	ThioTEPA-mercapturate fragment	
IVb	3.95	375.0692	$C_{11}H_{20}N_4O_3PS_2$ [Na$^+$]	0.5	ThioTEPA-mercapturate sodium adduct	
V	2.64	311.0776	$C_9H_{19}N_4O_2PS_2$ [H$^+$]	3.5	ThioTEPA-cysteinate	311; 268; 225; 181
VI	3.22	246.0326	$C_6H_{14}Cl_2N_3OP$ [H$^+$]	−1.6	1,2,-DichloroTEPA	246; 203; 167; 126; 80
VII	4.50	282.0105	$C_6H_{15}Cl_3N_3OP$ [H$^+$]	2.8	1,2,3-TrichloroTEPA	282; 203; 126; 80
VIII	2.52	220.0175	$C_4H_{12}Cl_2N_3OP$ [H$^+$]	0.9	DechloroethyltrichloroTEPA	220; 203; 141; 126; 80
IX	0.35	178.0171	$C_5H_9NO_4S$ [H$^-$]	−1.7	SCMC	178; 78
X	0.96	148.9912	$C_4H_6O_4S$ [H$^-$]	2.0	TDGA	149; 93
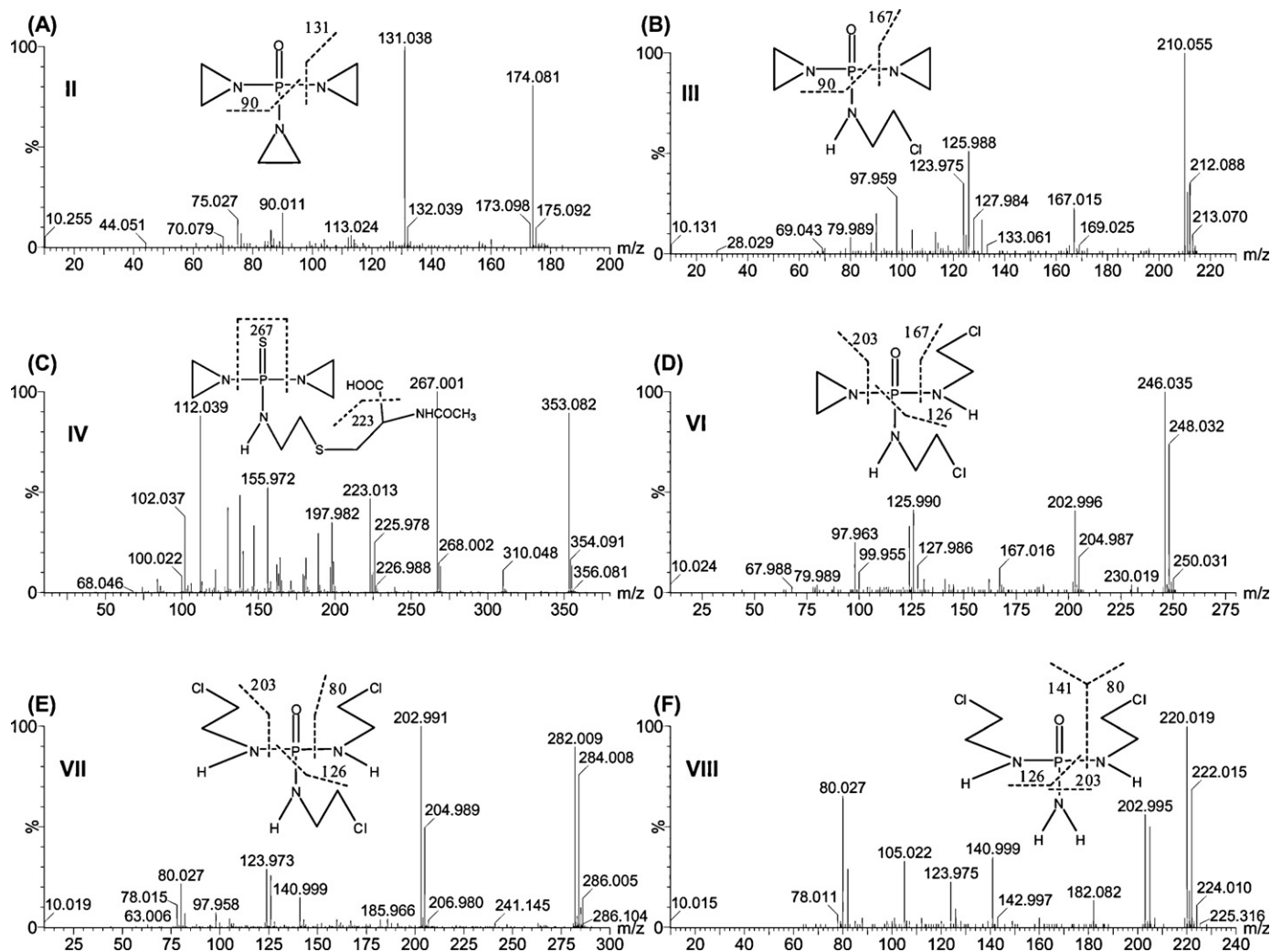
Fig. 3. Tandem MS and chemical structures of thioTEPA metabolites. (A) TEPA (II). (B) MonochloroTEPA (III). (C) ThioTEPA-mercapturate (IV). (D) 1,2-DichloroTEPA (VI). (E) 1,2,3-TrichloroTEPA (VII). (F) DechloroethyltrichloroTEPA (VIII). Note the chlorine isotope ratios depending on the presence of one (3:1), two (9:6:1) or three (27:27:9:1) chlorine atoms.

$(C_6H_{12}N_3OP)$ and monochloroTEPA $(C_6H_{13}ClN_3OP)$ suggested that metabolite VI was a ring-opened product from TEPA. Three chlorine isotope peaks m/z 246.035$^+$, 248.032$^+$ and 250.031$^+$ with the ion intensity ratio of 9:6:1 in its MS spectra confirmed that two aziridines had been opened to produce the corresponding chloroethylamine group. In its MS/MS fragmentation pattern (Fig. 3D), three ions m/z 202.996$^+$, 167.016$^+$, and 125.990$^+$ were generated by the loss of one aziridine, one chloroethylamine, and one aziridine and one chloroethylamine, respectively. According to the above inference, metabolite VI was identified as 1,2-dichloroTEPA, which was been reported to be a TEPA degradation product in the presence of sodium chloride at pH < 7 [35].

The new metabolite VII (1,2,3-trichloroTEPA) was calculated as $C_6H_{15}Cl_3N_3OP$ based on the accurate mass measurement, m/z 282.0105$^+$. Compared with the chemical composition of TEPA $(C_6H_{12}N_3OP)$ and monochloroTEPA $(C_6H_{13}ClN_3OP)$, it was concluded that metabolite VII was a ring-opened product from TEPA. As seen from the molecular ion peaks of VII (Fig. 3E), four chlorine isotope peaks m/z 282.009$^+$, 284.008$^+$, 286.005$^+$, and 288.233$^+$ were clearly visible. After the parent ions eliminated one chloroethylamine group (loss of 79 Da), the [M-79]$^+$ gave three chlorine isotope peaks m/z 202.991$^+$, 204.989$^+$, and 206.980$^+$. The observations suggested that three aziridines in VII had been

opened and transformed into the corresponding chloroethylamine group. In its MS/MS fragment (Fig. 3E), two ions m/z 202.991$^+$ and 125.983$^+$ were generated by the elimination of one chloroethylamine and two chloroethylamines, respectively. The ion m/z 80.027$^+$ was identified as a single chloroethylamine group. According to the above inference, metabolite VII was identified as 1,2,3-trichloroTEPA.

The new metabolite VIII (dechloroethyltrichloroTEPA) was calculated as $C_4H_{12}Cl_2N_3OP$ based on the accurate mass measurement, m/z 220.0175$^+$. Compared with the chemical composition of 1,2,3-trichloroTEPA $(C_6H_{15}Cl_3N_3OP)$, metabolite VIII was a N-dechloroethylation product from 1,2,3-trichloroTEPA by the loss of one chloroethyl group. This metabolic pathway was similar to the known reaction forming 2- or 3-dechloroethylifosfamide from ifosfamide [33]. Three chlorine isotope peaks m/z 220.019$^+$, 222.015$^+$ and 224.010$^+$ with the ion intensity ratio of 9:6:1 in its MS spectra were consistent with this conclusion. In its MS/MS fragmentation spectra (Fig. 3F), three ions m/z 202.995$^+$, 140.999$^+$, and 125.972$^+$ were generated by the loss of one amine group, one chloroethylamine, and one amine group and one chloroethylamine, respectively. The ion m/z 80.027$^+$ was identified as a single chloroethylamine group. Therefore, metabolite VIII was identified as dechloroethyltrichloroTEPA.

Table 2
Identified thioTEPA metabolites in mouse serum.

Symbol	RT (min)	Observed m/z	Formula	Mass error (ppm)	Identity
I	2.15	190.0582	$C_6H_{12}N_3PS$ [H$^+$]	7.3	ThioTEPA
II	1.51	174.0801	$C_6H_{12}N_3OP$ [H$^+$]	2.8	TEPA
III	1.56	210.0571	$C_6H_{13}ClN_3OP$ [H$^+$]	3.8	MonochloroTEPA
IV	2.19	353.0878	$C_{11}H_{21}N_4O_3PS_2$ [H$^+$]	1.9	ThioTEPA-mercapturate
VI	2.02	246.0334	$C_6H_{14}Cl_2N_3OP$ [H$^+$]	1.6	1,2,-DichloroTEPA
VII	2.47	282.0101	$C_6H_{15}Cl_3N_3OP$ [H$^+$]	1.4	1,2,3-TrichloroTEPA

Metabolite IX (SCMC) and X (TDGA) were most likely derived from chloroacetaldehyde, which can be generated from the side-chain of 1,2,3-trichloroTEPA [33]. SCMC and TDGA were detected in the negative mode. Their retention times and MS/MS fragmentation patterns were compared and their structures confirmed with authentic standards.

3.3. Identification of thioTEPA metabolites in serum

Although thioTEPA has been used clinically for decades, TEPA is the only identified metabolite in human and mouse serum. Here, metabolomic analysis was used to screen for and identify additional metabolites of thioTEPA in mouse serum samples. As shown in the scores scatter plot (Fig. 1B), serum samples from the thioTEPA-treated group were significantly separated from the control group. The thioTEPA parent ion (I), TEPA (II), monochloroTEPA (III), and thioTEPA-mercapturate (IV) were identified from the scores scatter plot (Fig. 1B). 1,2-DichloroTEPA (VI) and 1,2,3-trichloroTEPA (VII) were detected in the serum total ion chromatogram of thioTEPA-treated mice. All four metabolites except TEPA, are reported here for the first time (Table 2), and were identified by accurate mass measurement and MS/MS fragmentography.

3.4. Excretion of thioTEPA and its metabolites in urine

The absolute cumulative excretion of thioTEPA was quantitated against calibration curves constructed with authentic standard ($y = 0.172x - 1.673$, $r > 0.999$). As shown in Fig. 4A, the 24 h urinary excretion of unchanged thioTEPA was 0.27–0.51% of the total administered thioTEPA dose. Although standards of metabolites II–VIII were unavailable, their relative excretion in 24 h urine could be estimated based on their peak areas. In Fig. 4B and C, TEPA (II), monochloroTEPA (III), and thioTEPA-mercapturate (IV) were the major excreted metabolites in urine, while the excretion of the intermediate metabolite thioTEPA-cysteinate (V) was minor. The excretion of II, III and IV were about 10–15-fold in excess of parent compound urinary excretion. The majority of metabolites II–VIII, as well as of any unchanged thioTEPA, were excreted in the first 10 h following thioTEPA treatment.

The excretion of SCMC and TDGA in control and thioTEPA-treated mouse urine was quantitated against calibration curves constructed with authentic standards [$y = 1.027x - 0.435$, $r > 0.999$ (SCMC); $y = 0.973x + 0.951$, $r > 0.999$ (TDGA)] using triple quadrupole mass spectrometry. The 24 h urinary excretion of SCMC and TDGA in thioTEPA-treated mice was 1.4 ± 0.55 and
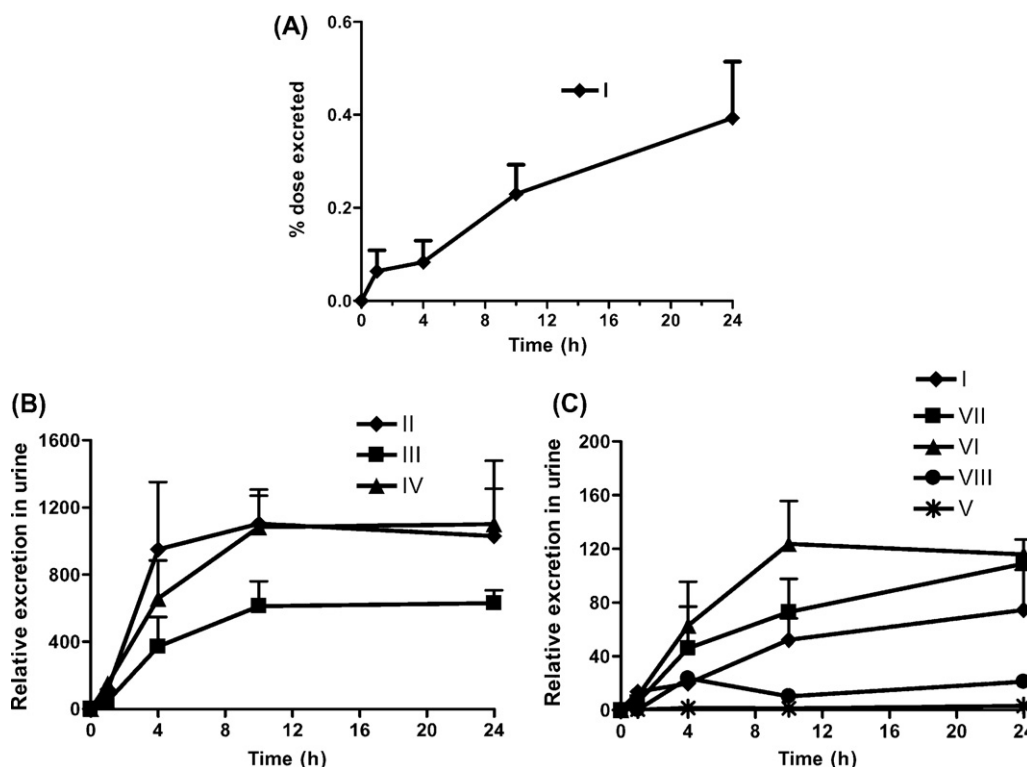

Fig. 4. Urinary excretion of thioTEPA and its metabolites from 0 to 24 h following the treatment of thioTEPA ($n = 4$). (A) Percent dose excretion of unchanged thioTEPA (I) at different time points. (B) Relative urinary excretion of thioTEPA metabolites II, III, and IV at different time points. (C) Relative urinary excretion of thioTEPA (I) and its metabolites V, VI, VII, and VIII at different time points.
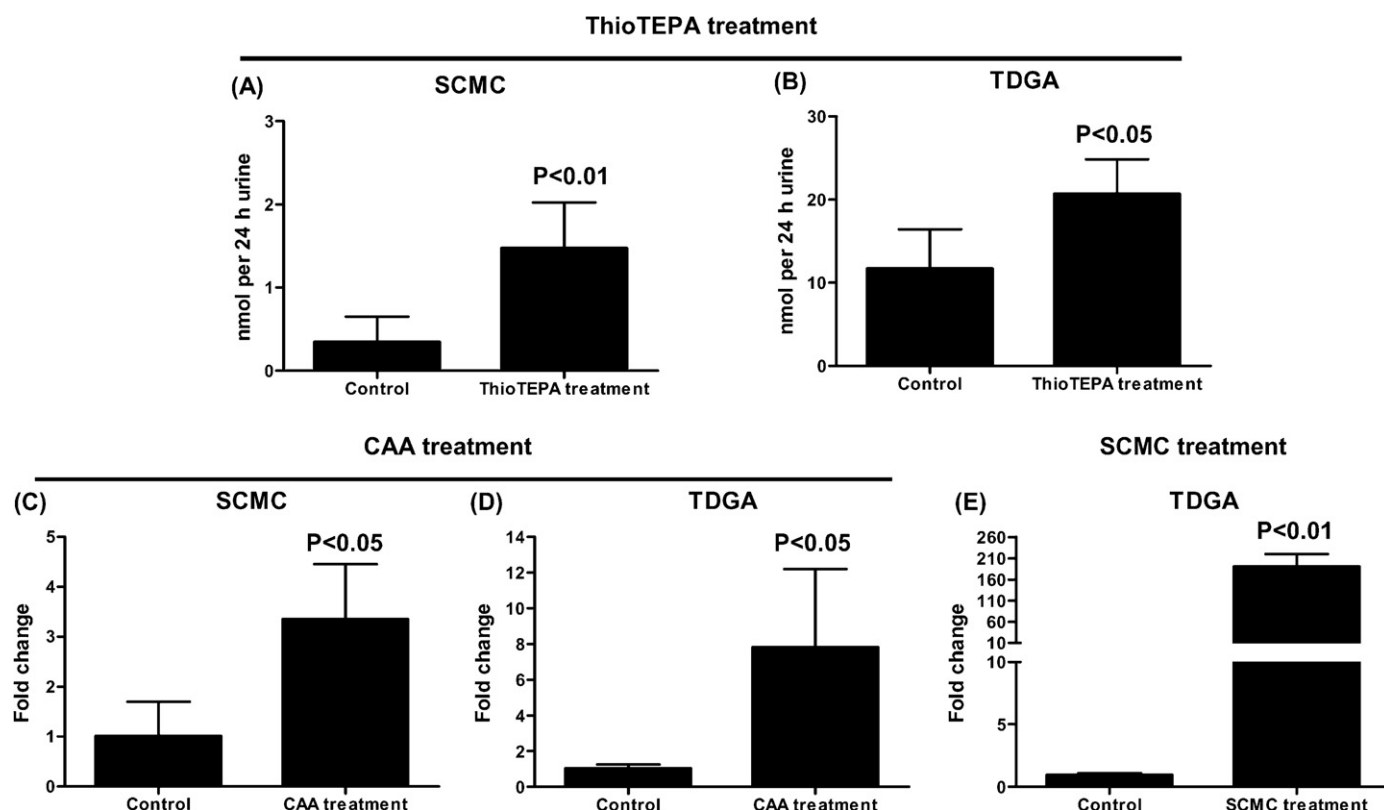
Fig. 5. The amount for SCMC and TDGA in 24 h mouse urine following the treatment of thioTEPA (50 mg/kg, approximately 1 mg of thioTEPA), CAA (2 mg/kg, approximately 0.04 mg of CAA), and SCMC (100 mg/kg, approximately 2 mg of SCMC). (A) The μmol/24 h of SCMC from control and thioTEPA-treated mice. (B) The μmol/24 h of TDGA from control and thioTEPA-treated mice. (C) The fold change of SCMC from control and CAA-treated mice. (D) The fold change of TDGA from control and CAA-treated mice. (E) The fold change of TDGA from control and SCMC-treated mice.

21.0 ± 4.2 nmol, respectively. As seen from Fig. 5A and B, SCMC and TDGA excretion was increased about 4-fold ($P = 0.0067$) and 2-fold ($P = 0.013$), respectively, in urine following thioTEPA treatment compared to control urine.

The relative excretion of SCMC and TDGA in control, CAA-treated, and SCMC-treated mouse urine was estimated by comparing their peak areas. The value was normalized by the peak area of creatinine. As seen from Fig. 5C and D, compared with control mice, SCMC and TDGA excretion in urine was increased about 3-fold ($P = 0.012$) and 7-fold ($P = 0.022$), respectively, following CAA treatment. As seen in Fig. 5E, an increase in urinary

excretion of TDGA was about 190-fold ($P < 0.0001$) following SCMC treatment compared with the control group.

3.5. Pharmacokinetics of thioTEPA and its metabolites in serum

The pharmacokinetic profile of thioTEPA (I) in mouse serum was determined for up to 10 h post-dose (Fig. 6A). The terminal elimination half-life was 38.6 ± 1.7 min, suggesting that thioTEPA is quickly cleared from mouse serum, which is similar to its elimination in humans [14]. In addition, the relative peak area-time profiles of thioTEPA metabolites (II, III, IV, VI and VII) were delineated (Fig. 6B).
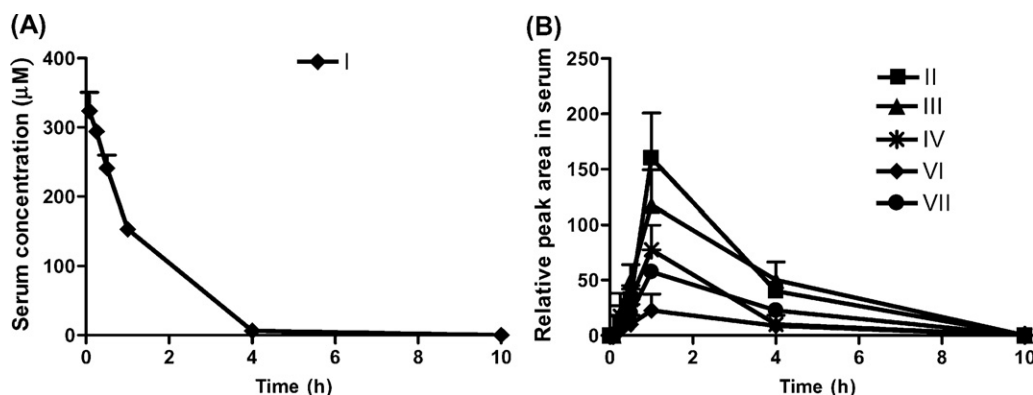

Fig. 6. The concentration-time profiles of thioTEPA and its metabolites in serum after intraperitoneal administration at 50 mg/kg of thioTEPA (approximately 1 mg of drug) in mice ($n = 4$). (A) Pharmacokinetic profile of thioTEPA (I) in mouse (from 0.08 to 10 h). (B) Relative peak area-time profiles of thioTEPA metabolites II, III, IV, VI, and VII in mouse (from 0.08 to 10 h).
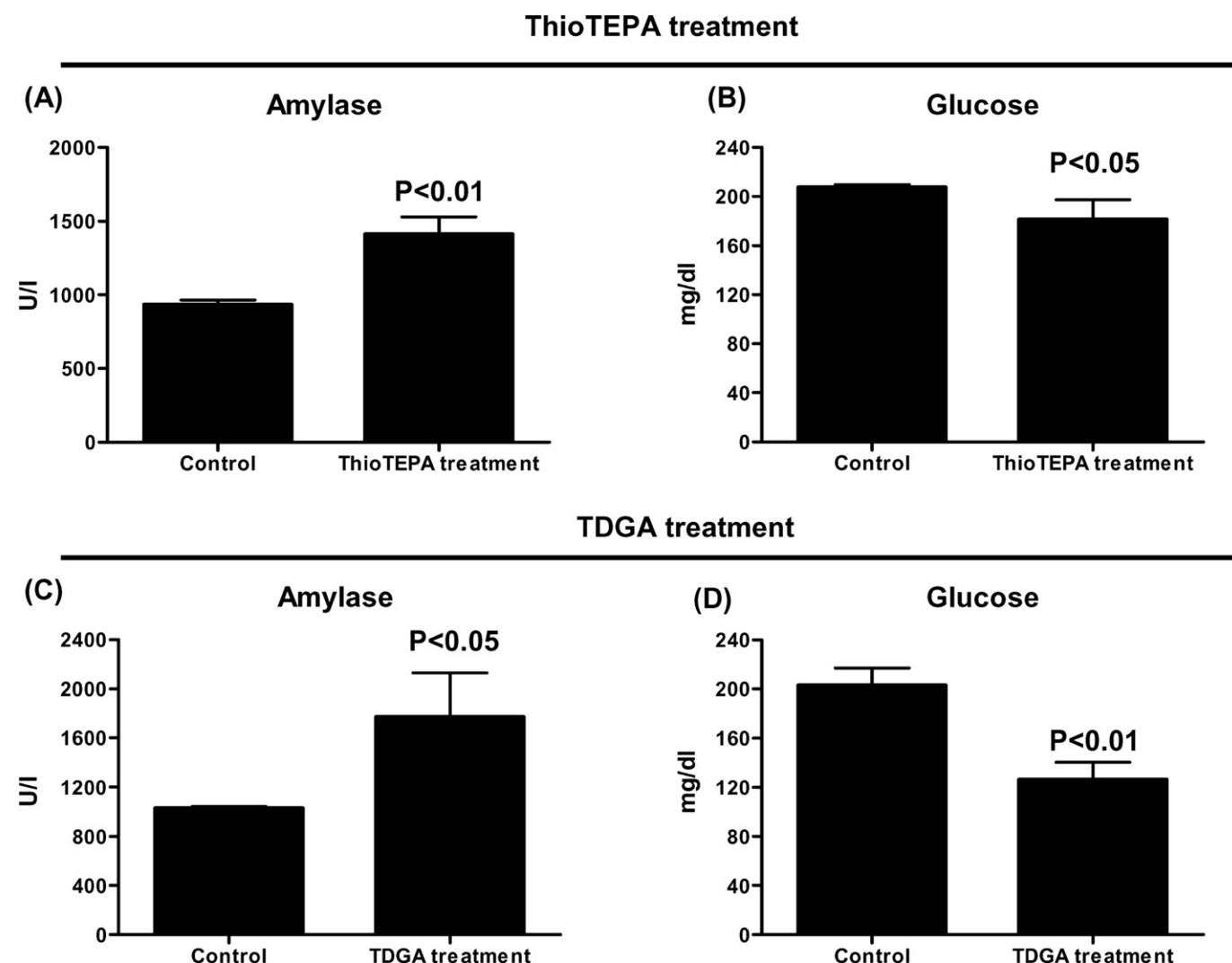
ThioTEPA treatment

Fig. 7. Serum chemistry of thioTEPA- and TDGA-treated mice. (A) Serum amylase in thioTEPA treatment and control mice. (B) Serum glucose in thioTEPA treatment and control mice. (C) Serum amylase in TDGA treatment and control mice. (D) Serum glucose in TDGA treatment and control mice.

These metabolites could first be detected at 15 min following parent drug administration, and achieved maximal concentrations (C_{max}) at approximately 1 h post-dose. Most of the parent thioTEPA and its metabolites were eliminated from mouse serum within the observation period of 10 h, consistent with the observed urinary excretion profile.

3.6. Serum chemistry

There was a significant difference in serum amylase and glucose levels following thioTEPA and TDGA treatment. As seen in Fig. 7A–D, serum amylase was increased in thioTEPA- and TDGA-treated mice compared with the control group, while serum glucose was decreased. The other serum parameters, such as ALT and ALP (data not shown), showed no significant differences.

4. Discussion

Metabolomics was applied to investigate the metabolism of thioTEPA in a mouse model *in vivo* and *in vitro*. In addition to the previously known metabolites TEPA (II), monochloroTEPA (III) and thioTEPA-mercapturate (IV), one conjugated metabolite (V) from thioTEPA and three ring-opened metabolites (VI, VII and VIII) were identified in the current study. Interestingly, the ring-opened

metabolites from thioTEPA showed a similar chemical structure to the alkylating agents ifosfamide and cyclophosphamide, which are widely used in the chemotherapy of various cancers. These metabolites can further eliminate the chloroethyl group by *N*-dechloroethylation, producing such metabolites as dechloroethyl-trichloroTEPA (VIII). Although CAA was not detected in the present study employing UPLC–ESI-QTOFMS, its downstream stable metabolites SCMC and TDGA were found in urine, and were quantitatively related to thioTEPA exposure. The major metabolic pathway of thioTEPA in the mouse *in vivo* is presented in Fig. 8. In addition, the urinary excretion of SCMC and TDGA were increased following CAA treatment (Fig. 5C and D). A previous study reported that TDGA was the major transformation metabolite of SCMC in humans [36]. Here, the excretion of TDGA was greatly increased in mouse urine after treatment with SCMC (Fig. 5E). These results suggested that CAA was first produced from the thioTEPA ring-opened metabolite (VII) by *N*-dechloroethylation, then CAA was transformed into SCMC, finally TDGA was generated from SCMC.

In the current study only one metabolite, TEPA, was detected after incubation with mouse liver microsomes, which was consistent with a previous result [10]. The nine metabolites of thioTEPA identified in mouse urine and serum suggest that thioTEPA can be transformed *in vivo* by non-cytochrome P450 pathways. Here, UPLC–ESI-QTOFMS-based metabolomics was a
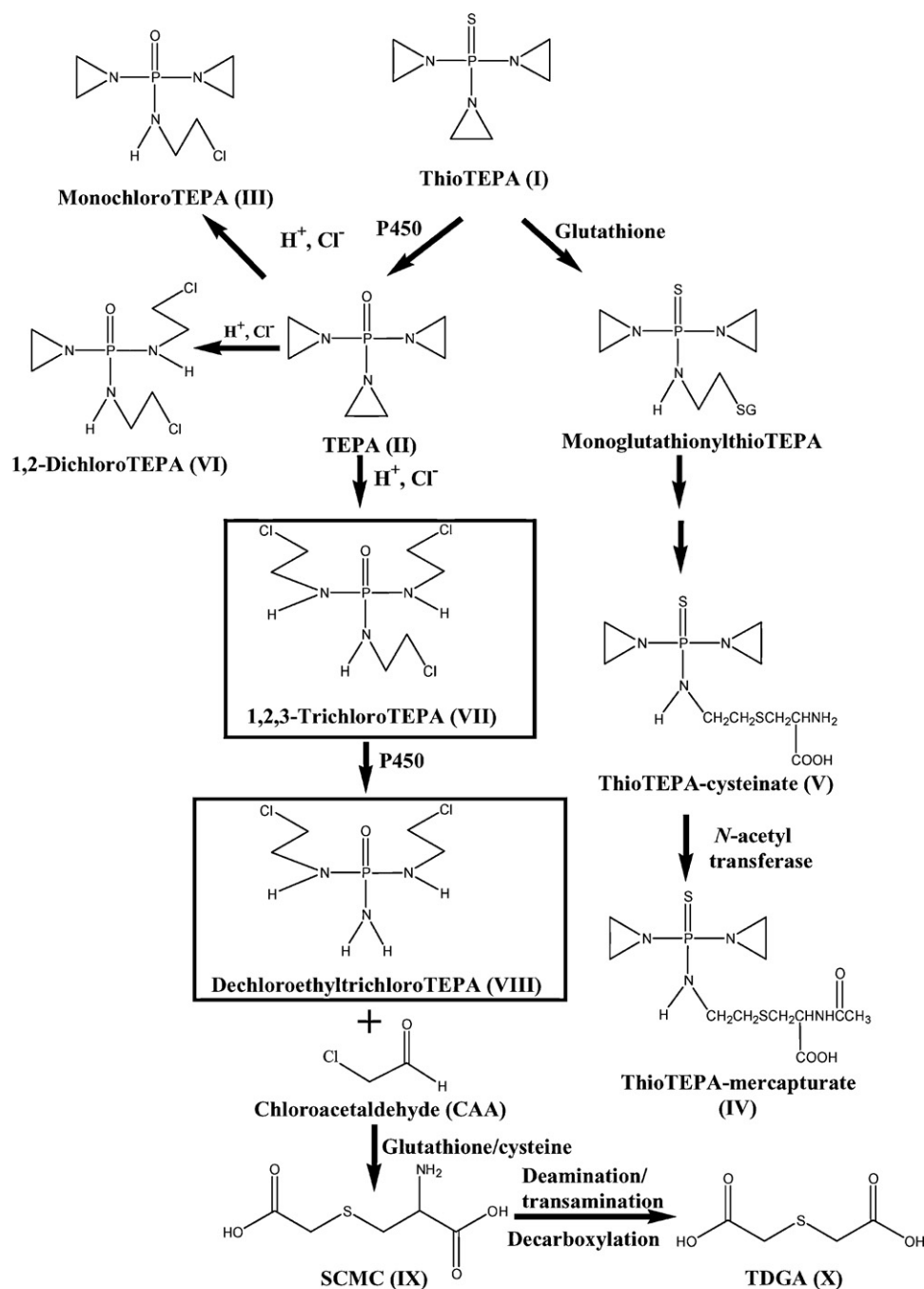
Fig. 8. Major *in vivo* ThioTEPA metabolic pathways. Boxed structures represent novel metabolites.

powerful tool for the identification of ring-opened metabolites (III, VI, VII, and VIII), especially for two novel metabolites 1,2,3-tricholoroTEPA and dechloroethyltrichloroTEPA (Figs. 1A and 3E–F). As seen in Figs. 2E–F and 4C, two novel metabolites, VII and VIII, show relatively low concentration in urine. This result emphasizes the value and sensitivity of UPLC–ESI-QTOFMS-based metabolomics for the identification of minor drug metabolites with potentially important functions *in vivo*. Previous studies [13,35,37] indicate that thioTEPA and TEPA can be degraded and generate chloro adducts in the presence of sodium chloride. Indeed, mono-, di-, and tri-chloro adducts of TEPA were identified in mouse urine and serum, while the corresponding chloro adducts of thioTEPA were not detected, potentially reflecting differences in the stability and chemical reactivity of thioTEPA and TEPA *in vivo*. It

was proposed that thioTEPA-mercapturate is generated from thioTEPA through the intermediate metabolites monoglutathio-nylthioTEPA and thioTEPA-cysteinate [8]. In this study, thioTEPA-cysteinate was detected in mouse urine thus confirming that thioTEPA-mercapturate was formed from thioTEPA by two intermediate metabolites *in vivo*.

Because thioTEPA bears three aziridines rings, the MS/MS fragmentation of thioTEPA and its alkylating metabolites share similar characteristics, and this was helpful for elucidating their chemical structures by mass spectrometry. The elimination of aziridine and chloroethylamine moieties, corresponding to the loss of m/z 43 and 80, respectively, was found to be a common reaction scheme. As seen from the MS spectra of thioTEPA and its metabolites (Fig. 3A–F), the MS/MS fragmentation of these

compounds can be explained with the elimination of aziridine, chloroethylamine, or both. Another interesting observation was that several metabolites containing the chloro adduct were generated. MS spectra of those metabolites show isotope peaks M + 2, M + 4 or M + 6, reflecting their possession of the chlorine associated stable isotopes ^{35}Cl and ^{37}Cl, and one, two or three Cl atoms, respectively.

ThioTEPA metabolites could first be detected in serum at 15 min post-dose in thioTEPA-treated mice, and serum concentrations were highest at 1 h. Seen in conjunction with the almost complete urinary excretion of the analytes over the 10 h observation period, the data suggest rapid renal clearance from the serum. ThioTEPA and all metabolites observed in this study reach the limit of detection in serum by 10 h post-dose, which may suggest similar serum clearance kinetics of metabolites and thioTEPA. The half-life of thioTEPA in the mouse was 38.6 ± 1.7 min, which is similar to a previous report [14]. This study also reported that TEPA can persist longer in the serum than thioTEPA, which was different from the present results ($t_{1/2}$ similar to that of thioTEPA) [14]. This difference may be due to the physiological or non-cytochrome P450 mediated species differences between humans and mice.

Previous studies reported that SCMC can activate the AMPA/kainate receptor and induce cellular acidification, and that TDGA can cause mitochondrial dysfunction [23,24]. Based on the known effects, it was proposed that increased serum concentrations of SCMC and TDGA following ifosfamide therapy may be responsible for ifosfamide-induced encephalopathy. In the present study, an increase of SCMC and TDGA was observed in thioTEPA-treated mice, suggesting a pathway for the generation of SCMC and TDGA from thioTEPA (Fig. 8). Interestingly, the generation of SCMC and TDGA from thioTEPA appears similar to that known for ifosfamide, which also undergoes *N*-dechloroethylation reactions. Clinical studies reported that patients developed serious encephalopathy following the administration of high dose thioTEPA [38]. Serum chemistry revealed that thioTEPA treatment can cause an increase in serum amylase and a decrease of glucose (Fig. 7A and B). As is known, increased serum amylase is an indicator of pancreatitis. Several cases in the clinic reported that encephalopathy can be observed in patients with pancreatitis [39,40]. The decrease of serum glucose suggested there was accelerated glucose metabolism *in vivo*, which could result in the consumption of thiamine [41,42]. It has been reported that thiamine deficiency is also another major cause of encephalopathy [43,44]. Interestingly, supplementation of thiamine can improve ifosfamide-induced encephalopathy in the clinic [45]. Additionally, TDGA treatment also caused an increase of amylase and decrease of glucose in serum (Fig. 7C and D), while SCMC treatment showed no significant differences for serum amylase and glucose in SCMC-treated and control mice (data no shown). It suggested the increased TDGA in thioTEPA treatment might be responsible for high-dose thioTEPA induced-encephalopathy. Therefore, further studies are needed to examine the toxicity mechanism of TDGA in relation to drug induced-encephalopathy.

As mentioned previously, thioTEPA is an alkylating agent and may form cross-links with DNA. It was reported that its metabolites TEPA, monochloroTEPA, and thioTEPA-mercapturate also possess alkylating activity [8]. 1,2-DichloroTEPA, 1,2,3-trichloroTEPA, and dechloroethyltrichloroTEPA were produced from thioTEPA by ring-opening of aziridine and the generation of the chloro adduct, suggesting that these metabolites might also have alkylating activity. ThioTEPA-mercapturate and thioTEPA-cysteinate, the conjugated metabolites of thioTEPA, might retain the alkylating activity of the parent [8]. As seen from Fig. 4B and C, TEPA, monochloroTEPA, and thioTEPA-mercapturate were the major metabolites *in vivo*, while 1,2-dichloroTEPA, 1,2,3-trichlor-

oTEPA, dechloroethyltrichloroTEPA, and thioTEPA-cysteinate were minor metabolites. It is estimated from these results that the total urinary combined excretion of TEPA, monochloroTEPA, and thioTEPA was about 40-fold greater than the excretion of thioTEPA itself (Fig. 4B and C), which is consistent with previous reports [16]. The combined total excreted amounts of thioTEPA, TEPA, monochloroTEPA, and thioTEPA-mercapturate accounts for 74% of the total alkylating activity [8]. The present study indicates that the sum of alkylating activity of thioTEPA and its metabolites may account for about 90% of the total generated alkylating activity. Therefore, theoretically, in addition to the metabolites identified in this study, additional metabolites may be formed *in vivo*. However, the discrepancy of 10% may at least in part reflect the loss of alkylating activity incurred if drug or metabolites interact with biomolecules such as DNA.

In summary, the metabolism of thioTEPA was comprehensively investigated in mice using a metabolomic approach. Nine metabolites in urine and five metabolites in serum, including two novel drug metabolites, were determined by this method. The generation of SCMC and TDGA from thioTEPA can be explained by the metabolic pathway proposed in this study. The *in vivo* transformation and serum/urine disposition of thioTEPA and its metabolites were systematically profiled in mice following a single i.p. application of thioTEPA at doses in line with previous work. Further studies are needed to investigate the toxicity mechanism of TDGA to aid in the clarification of high dose thioTEPA induced-encephalopathy.

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