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Simultaneous quantification of seven plasma metabolites of sulfur mustard by ultra high performance liquid chromatography–tandem mass spectrometry

Chunzheng Li, Jia Chen, Qin Liu, Jianwei Xie*, Hua Li*

Beijing Institute of Pharmacology and Toxicology, No. 27 Taiping Road, Haidian District, Beijing 100850, China

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ABSTRACT

Sulfur mustard (SM) is a hazardous chemical warfare agent that has been used in several military conflicts. SM is also considered as a major threat to civilians because of its existing stockpiles and easy production. Analysis of exposure biomarkers in biological samples collected from suspected victims is a useful tool for early diagnosis of SM poisoning. In this study, a sensitive and rapid quantitative method with ultra high performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed for simultaneous determination of seven SM plasma biomarkers, including its oxidative, hydrolysis and β-lyase metabolites. A simple one-step protein precipitation with acetonitrile–methanol (4:1) was used for sample preparation. A full validation was conducted with respect to specificity, linearity, recovery, matrix effect, precision, accuracy and stability. The lower limits of quantification for the seven metabolites ranged from 0.01 $\mu g\,L^{-1}$ to 5 $\mu g\,L^{-1}$. The intra-day relative standard deviation was less than 7.0%, and the interday deviation was less than 9.1%. The recoveries varied in the range from 82.8% to 118%. This method has been successfully applied to a toxicokinetic study for obtaining the plasma profiles of seven metabolites in SM-exposed rats, following a single subcutaneous dose of 3.3 $mg\,kg^{-1}$. All the targeted compounds were detected in rat plasma. *bis*-*β*-Chloroethyl sulfoxide (SMO), thiodiglycol (TDG), thiodiglycol sulfoxide (TDGO), 1,1′-sulfonylbis-[2-S-(N-acetylcysteinyl)ethane (SBSNAE), 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE) were found to be the major metabolites in rat plasma. The time windows for the detection of these metabolites were varied in the range of 5 min to 48 h after exposure. The method provides a useful tool for short-term diagnosis of SM poisoning.

1. Introduction

Bis(2-chloroethyl)sulfide, commonly known as sulfur mustard (SM), is one of the most hazardous chemical warfare agents. It was initially deployed as a chemical weapon in conflicts in 1917, 95 years ago. The most recent confirmed use was during the Iran–Iraq war in late 1980s [1–3]. SM was also a predominant agent found in the chemical weapons abandoned in China by the Japanese army after World War II [4,5]. These abandoned weapons were either hidden in mountain caves or buried in the earth. Some of these weapons and containers holding chemical warfare agents have deteriorated and the leaking-out agents have caused repeated incidents of casualty and environmental contamination [4], which poses a great health threat to civilians.

SM is a potent vesicant agent producing severe blistering when contacted with skin. It also causes respiratory tract damage, eye lesions and bone marrow depression. The injury caused by SM is characterized by an initial asymptomatic latent period of 1–12 h that precedes lesion development [6]. This latent period may cause delayed diagnosis and treatments, particularly for accidentally injured civilians. One strategy for early and reliable diagnosis of SM poisoning is analysis of its biomarkers in biological samples collected from suspected victims [7,8].

A number of SM biomarkers have been studied in previous researches and used for monitoring SM exposure and retrospective analysis, including free metabolites and covalent adducts with macromolecules, such as proteins and DNA [8–10]. The urine metabolites of SM in rats were initially reported by Black et al. [11] and ten metabolites were identified, including oxidative and hydrolysis metabolites *bis*-*β*-chloroethyl sulfoxide (SMO), thiodiglycol (TDG) and thiodiglycol sulfoxide (TDGO), glutathione-derived metabolite 1,1′-sulfonylbis-[2-S-(N-acetylcysteinyl)ethane (SBSNAE), the β-lyase metabolites 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE). Some of these metabolites (TDG, TDGO, SBMTE and

* Corresponding authors. Tel.: +86 10 88270677; fax: +86 10 68225893.
E-mail addresses: xiejw@nic.bmi.ac.cn (J. Xie), amms_hli@126.com (H. Li).

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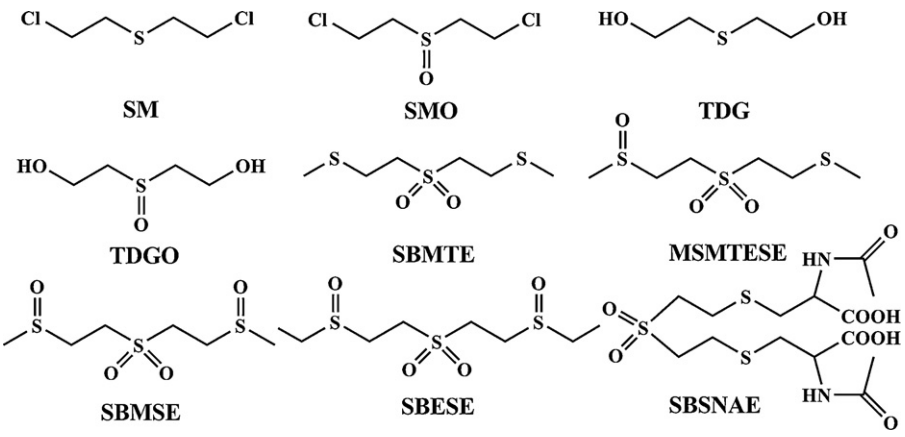

Fig. 1. Structures of SM, metabolites and internal standard.

SBSNAE) were also detected in human urine following accidental or deliberate exposures [7,10]. For the purpose of early diagnosis of SM intoxication, TDG, TDGO, SBSNAE and β-lyase metabolites in plasma or urine were considered as appropriate exposure biomarkers, as they exist in these biological specimens for hours or even days after exposure [12,13]. The analysis procedures for the metabolites are much simpler and less time consuming when compared to those involving SM adducts. The structures of SM and the metabolites are presented in Fig. 1.

Accurate measurement of aforementioned biomarkers can provide information of SM exposure levels. Several analytical methods have been established for measuring some of these metabolites in urine samples. The quantitative analysis of TDG and TDGO was conducted mainly using gas chromatography–tandem mass spectrometry (GC–MS/MS) following derivatization [14–17]. The first quantitative method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for TDG and TDGO was recently developed in our laboratory [18]. The analysis of β-lyase metabolites with GC–MS or GC–MS/MS required an additional procedure to reduce both SBMSE and MSMTESE to a single analyte 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE), using titanium trichloride [15,19]. To simplify the analytical procedures for β-lyase metabolites quantification, LC–MS/MS methods were developed and validated [13,20,21]. The above mentioned methods have been used for quantification of SM biomarkers in urine samples. However, the analytical methods for analysis of plasma biomarkers were rarely reported. A LC–MS/MS quantitative method was published recently for analyzing the plasma concentration of a single compound (SBSNAE) [22], but no application of the method was reported.

Analysis of blood or plasma samples provides critical qualitative and quantitative information for early diagnosis of SM exposure, as biomarker concentrations in these samples can directly reflect the real-time exposure level in the circulation which is more relevant to the toxicological effects of the agent. However, the plasma SM biomarkers were not thoroughly studied and very limited information was available. In this study, a rapid and sensitive UPLC–MS/MS method was established for simultaneous quantitation of 7 SM metabolites in plasma. The method was fully validated to meet the bioanalytical requirements. It is simple, rapid, sensitive and could quantitatively determine both oxidative/hydrolysis metabolites (TDG, TDGO and SMO) and glutathione-derived metabolites (SBSNAE, SBMTE, MSMTESE and SBMSE) in one injection. TDG and TDGO are directly analyzed without derivatization. This novel method has been successfully applied to a toxicokinetic study. The plasma profiles of these metabolites were determined in SM-exposed rats. The plasma levels of each biomarker were assessed from its concentration–time course, and time windows for quantitative detection were obtained. The results indicated that SMO, TDGO, SBSNAE, MSMTESE and SBMSE may be used as plasma biomarkers for early diagnosis of SM exposure. The method is suitable for early monitoring of SM poisoning, in the situation of a mass-casualty terrorist attack, as well as in the cases of accidental injury caused by abandoned SM chemical weapons in China.

2. Experimental

2.1. Materials and reagents

Thiodiglycol (TDG) was purchased from Sigma–Aldrich (ST. Louis, MO, USA). Thiodiglycol sulfoxide (TDGO), 2,2′-dichloroethyl sulfoxide (SMO), 1,1′-sulfonylbis[2-*S*-(*N*-acetylcysteinyl)ethane] (SBSNAE), the β-lyase metabolites 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE), 1-methylsulfinyl-2-[2-(methylthio) ethylsulfonyl]ethane (MSMTESE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE) were synthesized in house according to previously published procedures [23,24]. The purities of these reference substances were determined to be >95% by NMR spectrometry and either LC–MS or GC–MS. 1,1′-Sulfonylbis[2-(ethylsulfinyl) ethane](SBESE), an analog of the β-lyase metabolites, was also synthesized (purity >95%, NMR, high resolution-MS) and used as the internal standard (I.S.). HPLC pure grade of methanol and acetonitrile were obtained from J&K Scientific LTD (Beijing, China), and ammonium formate was the product of Beijing Chemical Reagent Company (Beijing, China). High-purity water was generated by a Milli-Q A10 filtering system (Millipore, Billerica, MA, USA).

2.2. Instrumentation

Analysis was performed on a system consisting of an Acquity UPLC comprising a binary solvent manager and a sample manager (Waters, Manchester, UK), and a Qtrap 5500 tandem mass spectrometer (AB Sciex, Framingham, MA, USA). The data acquisition and processing were carried out by AB Sciex Analyst 1.5.1. Equipment used for sample preparation included 5418 high speed centrifuge (Eppendorf, Hamburg, Germany) and RVC 2-33CD rotational-vacuum-concentrator (Christ, Osterode, Germany).

2.3. UPLC–MS/MS conditions

The chromatographic separation of the seven analytes was achieved on a Zorbax Eclipse Plus C_{18} column (100 mm × 3.0 mm i.d., 1.8 μm particle size) from Agilent (Santa Clara, CA, USA). The

column temperature was maintained at 40 °C. A binary gradient program was applied with the mobile phase A of water–methanol (99:1, v/v) containing 5 mmol L^{-1} ammonium formate and B of methanol–water (95:5, v/v) containing 5 mmol L^{-1} ammonium formate. The elution gradient started at 100% of mobile phase A for 1 min, the mobile phase B was then linearly ramped from 5% (1.5 min) to 17% (3.4 min), and continuously raised to 44% at 9 min then kept constant at 80% for 1 min before being returned to the initial conditions. The column was re-equilibrated for 3 min with the initial mobile phase solvent. The flow rate was set at 0.3 mL min^{-1} and total runtime was 13 min. The injection volume was 2 μL.

The mass spectrometric detection was performed using positive ion electrospray MS/MS in selected reaction monitoring (SRM) mode. The transitions of the analytes were selected based on their full scan mass spectra obtained by directly infusing standard solutions into the mass spectrometer ESI source. For each transition, the optimized parameters declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), ionspray voltages (IS) and collision gas (CAD) were optimized and shown in Table 1. The first SRM transition for each molecule was used for quantification, while the second transition was monitored only for the confirmation of molecular identification. Other source conditions were as follows: 550 °C for source temperature, 379 kPa for both Gas 1 and Gas 2, and 241 kPa for curtain gas.

2.4. Matrix-matched calibration

Matrix-matched calibration curves were obtained and used for quantification. Blank plasma samples were taken from the rats (n = 33) with no SM exposure history and tested prior to the study. A low background level of TDGO at 0.2–0.8 μg L^{-1} was detected in all 33 blank samples, and a relatively higher level (3–10 μg L^{-1}) of TDG was observed in the plasma samples from 19 rats. The blank plasma samples used for preparation of calibration standards were selected by the preset criteria of TDG-free and TDGO level below 0.3 μg L^{-1}.

A composite stock solution (10 mg L^{-1} each) for all the analytes except SMO was prepared by mixing equal volume of individual standard in acetonitrile. The stock solution for SMO was prepared separately to avoid the possible interaction with other analytes. The stock solutions were divided into small portions and stored at −20 °C. The working solutions with a series of concentrations were made freshly before analysis by mixing two standard solutions and further diluting with acetonitrile accordingly.

Calibration standards were prepared by spiking appropriate working solutions containing TDG, TDGO, SBMSE, SBMTE, MSMTESE, SBSNAE and SMO into 50 μL blank rat plasma. The quality control samples were made in the same manner and used for all the validation tests. The concentrations of the calibration standards and the quality control samples were decided for each analyte according to their MS sensitivity.

2.5. Sample preparation

A simple one-step protein precipitation in a mixed solvent of acetonitrile–methanol (4:1) was used for sample preparation. An aliquot of 50 μL plasma sample was mixed with 200 μL solvent containing 10 μg L^{-1} I.S. to precipitate plasma proteins. After vortexing for 60 s, the mixture was centrifuged at 14,000 rpm for 15 min. The supernatant was then transferred to a clear vial and evaporated to dryness at 50 °C in a rotational-vacuum-concentrator. The residue was then dissolved in 100 μL 10% acetonitrile aqueous solution followed by centrifugation at 14,000 rpm for 5 min. The supernatant (2 μL) was analyzed using UPLC–MS/MS.

2.6. Validation study

The UPLC–MS/MS method was fully validated as a quantitative confirmatory method for specificity, linearity, precision, recovery, and stability [25]. The process for quantification was based on peak areas response of the most intense SRM transition divided by the peak area response of the corresponding internal standard.

2.6.1. Specificity

The specificity of the method was checked by analyzing the blank, spiked and real plasma samples. A comparison of chromatograms was made among blank plasma, blank sample spiked with analytes, and real plasma samples taken from the SM exposed rats to confirm the suitability of the chromatographic elution condition. Carryover was investigated by analyzing a solvent blank (10% acetonitrile aqueous solution) and a sample blank (negative control) before and after each analytical run during method validation and routinely in each analytical sequence.

2.6.2. Linearity and sensitivity

Calibration curves were generated for the seven analytes within the concentration ranges, with three replicates at each concentration. The linearity of the method was evaluated using $1/x^2$ weighted least squares linear regression analysis of the matrix-extracted calibration curves, by plotting the peak area ratio of individual analyte to internal standard versus concentration. The limit of detection (LOD) was set at the signal-to-noise ratio of 3 (S/N = 3). The lower limit of quantification (LLOQ) was decided as the lowest concentration on the calibration curve which met the criteria of precision not more than 20%, accuracy within ±20%, and S/N > 5 [25].

2.6.3. Precision and accuracy

The intraday precision and accuracy of the method were determined by analyzing the six replicates on the same day at three QC levels, and the interday variation was determined in six consecutive days at two concentration levels. The precision and accuracy were expressed as relative standard deviation (RSD) and relative error (RE), respectively.

2.6.4. Recovery and matrix effect

Recoveries of the seven compounds from plasma were assessed by analyzing spiked blank plasma samples at three QC concentrations in six replicates. To evaluate the matrix effects, the post-extraction spike procedure [26] was applied. The blank plasma samples from six rats were processed as per the procedure detailed in Section 2.5, then spiked with the analytes and I.S. The peak areas of these post-extraction spiked samples were compared with those of the standard solutions at the same concentrations to calculate the matrix effects (ME %).

2.6.5. Stability

The stability of the SM metabolites in both plasma and processed samples was investigated at two concentration levels (5 μg L^{-1} and 50 μg L^{-1}) in three replicates. The freeze–thaw stability was studied after three cycles consisting of freezing samples at −80 °C for 24 h followed by thawing at room temperature. Long-term stability was assessed after storing samples at −80 °C for 35 days. The stability of the processed samples in the autosampler tray was measured at 4 °C for 24 h to ensure the integrity of the analytes during the course of an analytical run.

2.7. Toxicokinetic study

Six specific pathogen-free (SPF) grade male Sprague–Dawley rats (275 ± 25 g) were obtained from the Beijing Experimental

Table 1
Optimized parameters of SRM transitions for the biomarkers.

Compound	Transition	DP (V)	CE (V)	CXP (V)	IS (V)	CAD (V)
SMO	175 → 63[a]	120	26	16	1800	9
	175 → 57					
TDG	123 → 105	50	9	10	1750	6
	105 → 87					
TDGO	139 → 77	50	18	10	1450	9
	139 → 63		22	8		
SBMTE	215 → 75	50	15	16	1450	6
	215 → 119		23	10		
MSMTESE	231 → 75	50	20	10	1450	6
	231 → 167		15	16		
SBMSE	247 → 183	50	15	16	1450	9
	247 → 119		29			
SBSNAE	445 → 357	50	29	28	1750	6
	445 → 130		38	10		

[a] Quantitative ions.

Animal Center (Beijing, China). The animals were allowed to acclimatize for at least one week prior to the study. The animal experiment was conducted in the Beijing Center for Drug Safety Evaluation, in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Center, which is in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The injection solution of SM (0.3%, *V/V*) was prepared immediately before dosing, by dissolving SM in 2-propanol (less than 10% of the final concentration) followed by diluting with saline. The rats were subcutaneously injected with the solution at the exposure dose of 3.3 mg kg^{-1}. Blood samples (120 μL each) were collected before administration and at 5, 15, 30 min, 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after dosing. The plasma samples were obtained by immediate centrifugation of the blood samples at 3000 rpm for 10 min, and then stored at −80 °C until analysis.

The concentrations of the SM metabolites in rat plasma were quantitatively determined by the established UPLC–MS/MS method. Each analytical run for real samples was accompanied by a freshly plotted calibration curve, as well as a set of QC samples to verify the quality of the run.

2.8. Data analysis

The data were analyzed using Analyst® (version 1.5.1, AB Sciex, Framingham, MA, USA). The chromatographic peaks were smoothed prior to integration. The toxicokinetic parameters of the SM metabolites were obtained by fitting the concentration–time data to the non-compartmental model of WinNonlin® software (version 5.2.1, Pharsight Corp, CA, USA).

3. Results and discussion

3.1. Optmization of sample preparation

This method was intended for rapid quantification of the plasma biomarkers after SM exposure. A simple one step protein precipitation procedure was chosen to shorten the sample preparation time. The optimization of the procedure was obtained after testing several precipitating solvents and different solvent compositions. The results with the best recoveries and lowest matrix effects for all seven analytes were achieved with a mixed solvent containing 80% acetonitrile and 20% methanol at the solvent to plasma ratio of 4:1. High speed centrifugation at 14,000 rpm twice helped to remove tiny particles and increase the service life of the column. Instead of the mobile phase, 10% acetonitrile aqueous solution was selected as the reconstituting solvent. TDGO showed a better peak shape and appropriate retention time when treated with this solution than other solvents, such as pure methanol or any solution with acetonitrile concentration higher than 10%.

3.2. UPLC–MS/MS analysis

The internal standard was a key for accurate quantification of multiple analytes in one run. In this case, the ideal internal standard should have a chromatographic behavior and a MS ionization property compatible with both hydrolysis metabolites and β-lyase metabolites. A number of compounds were tested in this study and SBESE, an analog to the β-lyase metabolites stood out. SBESE has an appropriate retention time and eluted in the middle of the seven analytes (Fig. 2b). It also shows a stable and efficient ionization property.

Under the current chromatographic conditions, the seven analytes and the I.S. were well separated in 8 min, except TDG and SBSNAE. They co-eluted from the column at 4.1 min (Fig. 2b), even after several attempts for adjusting mobile phase compositions and gradient programs. However, with different SRM transitions, the quantitative measurement of TDG and SBSNAE was not affected, and the good precision and accuracy for both compounds were observed. The LLOQ of TDG under the current UPLC–MS/MS condition was found to be 5 μg L^{-1} even after the mass spectrometric parameters were optimized in favor of TDG scan. Considering its relatively higher background level in rat or

Table 2
Linearity and LLOQ of the method.

Compound	Equation	r^2	Range	LLOQ (μg L^{-1})
SMO	$Y = 0.0127X + 0.0005$	0.9998	0.05–500	0.05
TDG	$Y = 0.0078X + 0.0082$	0.9946	5–500	5
TDGO	$Y = 0.0111X + 0.0188$	0.9990	0.5–500	0.5
SBMTE	$Y = 0.1253X + 0.0005$	0.9978	0.05–500	0.05
MSMTESE	$Y = 0.1424X + 0.0004$	0.9948	0.01–500	0.01
SBMSE	$Y = 0.0389X + 0.0002$	0.9970	0.01–500	0.01
SBSNAE	$Y = 0.0026X - 0.0003$	0.9964	1–500	1

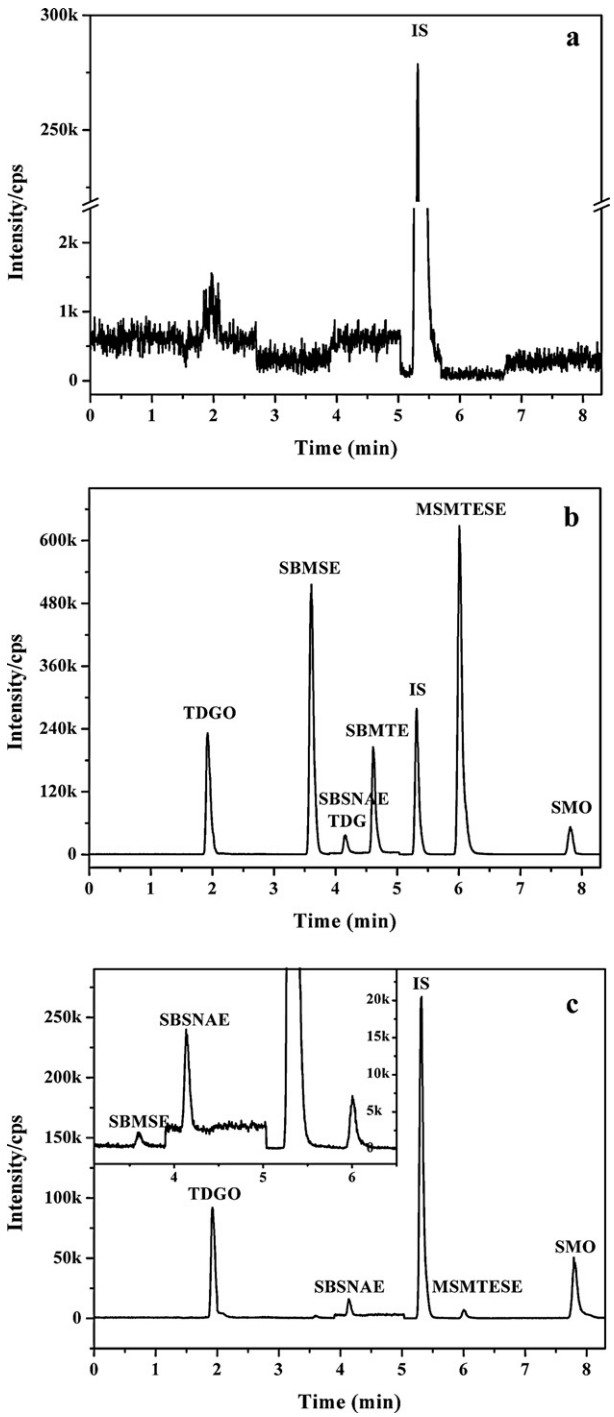

Fig. 2. Representative SRM chromatograms of (a) blank plasma spiked with I.S.; (b) blank plasma spiked with 7 metabolites; (c) real rat plasma sample taken after 4 h of SM exposure.

human plasma, the sensitivity of TDG in the method was sufficient for determining the plasma concentrations.

3.3. Method validation

The specificity of the UPLC–MS/MS method was assessed by comparing chromatograms among blank plasma, standard spiked samples, and the real plasma samples taken from SM exposed rats (Fig. 2). The blank plasma used for this study was pre-screened for the background levels of the SM biomarkers. The TDG free samples with the TDGO level below 0.3 $\mu g\,L^{-1}$ were selected and used. The representative SRM chromatogram of the blank samples (Fig. 2a) showed a weak signal of TDGO only. No significant endogenous interferences were detected at the peak regions of the analytes and the I.S.

Calibration curves with at least seven points were plotted over varied concentration ranges for the seven metabolites. The peak area ratio of the analyte versus the internal standard was calculated for each point. The curves were generated by linear regression analysis using a weighting coefficient of $1/x^2$. The mean regression equations from three replicate calibration curves, linear ranges and LLOQs are listed in Table 2. It is shown that all the biomarkers could be detected at a LLOQ of less than1 $\mu g\,L^{-1}$ except for TDG (5 $\mu g\,L^{-1}$).

The intra-day precision and accuracy were determined at three QC levels in six replicates. The precision (RSD) for all the analytes was below 7.0%. The accuracy (RE) was in the range from −14.0 to 14.0% (Table 3). The inter-day precision and accuracy were assessed at two concentration levels (5 and 50 $\mu g\,L^{-1}$) in six consecutive days. The RSD was shown to be below 6.4% and the RE within the range of −11.2 to 12.2% (Table 4). The results indicated that the precision and accuracy of this method met the criteria recommended by FDA [25], which requires 15% deviation of standards from nominal concentrations other than LLOQ that should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%.

The recovery and the matrix effect of the SM biomarkers were assessed at low, medium and high concentration levels, and the data are presented in Table 5. The recoveries were found to be above 82% for all the analytes. The matrix effect was within the range of 75–118%, except for TDG and SBSNAE at the low concentration, where 135% and 151% were observed respectively. The plasma matrix had an enhancement effect on the signals of TDG and SBSNAE, which might be caused by the co-elution of these two compounds. However, the matrix effects of these two were consistent among the samples from different sources. RSD and RE were all within the accepted range even at the low concentration, which indicated that the precision and accuracy were not compromised.

The stability of the SM biomarkers in plasma was studied at two concentration levels of 5 and 50 $\mu g\,L^{-1}$. The variation between the freshly prepared samples and the samples stored under different conditions are listed in Table 6. The RSD values were all below 15%, except TDG after long term storage. All the analytes demonstrated the sufficient stabilities in the processed samples after three freeze–thaw cycles. SMO, SBMTE, MSMTESE, SBMSE and SBSNAE were also stable after storage at −80 °C for 35 days, but not for TDG and TDGO. Further study indicated that TDG and TDGO were stable in the first 10 days while stored at −80 °C, and the conversion between these two compounds in plasma was observed afterwards. This indicated that, for accurate quantification of TDG and TDGO, plasma samples should be analyzed within 10 days after sample collection.

3.4. Toxicokinetics of SM metabolites

The validated method has been successfully used for the toxicokinetic study in the rats following a single subcutaneous dose (3.3 mg kg^{-1}) of SM. The plasma profiles of the SM metabolites are shown in Fig. 3 and the major toxicokinetic parameters are summarized in Table 7. All seven metabolites were detected in the plasma of the SM exposed rats. However, remarkable differences in both plasma level and time course were observed between the oxidative/hydrolysis metabolites (SMO, TDG and TDGO) and the glutathione-derived metabolites (SBMTE, MSMTESE, SBMSE and SBSNAE). The oxidative/hydrolysis metabolites appeared in the plasma very rapidly. The average plasma levels of 264.60 ± 111.46, 460.8 ± 113.6 and 14.02 ± 5.86 $\mu g\,L^{-1}$ were determined respectively for SMO, TDG and TDGO at the first sampling point (5 min).

Table 3
Intra-day precision and accuracy of the method ($n = 6$).

Compounds	L[a]		M		H	
	RE%	RSD%	RE%	RSD%	RE%	RSD%
SMO	−6.25	3.74	3.90	4.93	6.34	1.86
TDG	12.00	6.46	−4.29	3.62	7.94	1.75
TDGO	14.00	4.17	10.41	1.63	−7.18	2.80
SBMTE	−6.00	4.68	8.78	3.70	−4.46	2.24
MSMTESE	−3.33	2.72	3.16	2.20	4.90	2.36
SBMSE	3.33	6.48	−6.09	4.93	6.85	3.45
SBSNAE	−14.00	6.97	−3.19	5.64	10.79	3.04

[a] Low, medium and high concentrations were selected according to the linear range of each metabolite.

Table 4
Inter-day precision and accuracy of the method ($n = 6$).

Compounds	5 μg L^{-1}		50 μg L^{-1}	
	RE%	RSD%	RE%	RSD%
SMO	3.60	2.40	−3.44	2.98
TDG	−11.20	6.36	2.28	5.85
TDGO	−3.80	2.21	−8.50	2.15
SBMTE	−8.40	2.47	6.24	2.44
MSMTESE	2.20	3.54	−2.98	2.48
SBMSE	4.80	2.78	−4.12	1.63
SBSNAE	12.20	4.72	3.42	3.27

Table 5
Matrix effect and recovery of the method.

Compounds		SMO	TDG	TDGO	SBMTE	MSMTESE	SBMSE	SBSNAE
Matrix effect	L	86%	135%	93%	112%	88%	75%	151%
	M	118%	118%	77%	101%	100%	85%	110%
	H	110%	93%	80%	88%	107%	83%	112%
Recovery	L	91.8%	118%	105%	104%	108%	84.1%	103%
	M	103%	115%	113%	104%	118%	96.4%	113%
	H	95.8%	101%	106%	82.8%	106%	95.0%	105%

Table 6
Concentration variation of the metabolites in the stability study ($n = 6$).

Compound	Stability (RSD%)					
	Freeze–thaw		Post processing		35-day (−80 °C)	
	L[a]	H[b]	L	H	L	H
SMO	4.84	13.79	6.64	3.59	3.51	4.85
TDG	8.37	10.70	8.88	7.34	24.86	14.79
TDGO	5.00	6.93	5.93	3.63	10.91	8.94
SBMTE	4.94	11.54	5.96	4.05	2.57	4.99
MSMTESE	3.37	8.29	9.10	5.71	12.32	8.77
SBMSE	7.05	14.12	3.99	3.27	4.76	4.88
SBSNAE	3.22	12.89	7.11	3.19	10.16	6.42
SBESE	0.34	0.37	0.22	0.30	0.35	0.30

[a] 5 μg L^{-1}.
[b] 50 μg L^{-1}.

Table 7
Toxicokinetic parameters of the SM metabolites ($\mu \pm \sigma$, $n = 6$).

Compound	Parameter				
	C_{max} (μg L^{-1})	T_{max} (h)	$AUC_{(0-t)}$ (h μg L^{-1})	$T_{1/2}$ (h)	MRT (h)
SMO	435.76 ± 48.64	0.8 ± 0.4	1058.10 ± 136.72	0.6 ± 0.1	1.6 ± 0.2
TDG	538.0 ± 167.8	0.2 ± 0.1	343.4 ± 123.8	0.4 ± 0.1	0.5 ± 0.1
TDGO	82.52 ± 41.96	0.5 ± 0.0	173.74 ± 52.96	2.5 ± 0.9	2.3 ± 0.3
MSMTESE	7.08 ± 1.38	8.0 ± 0.0	57.80 ± 10.64	7.5 ± 1.5	11.2 ± 0.4
SBMSE	1.98 ± 0.50	8.0 ± 0.0	16.90 ± 6.00	5.7 ± 0.9	11.6 ± 0.6
SBSNAE	8.56 ± 2.54	3.7 ± 0.5	33.72 ± 9.22	1.4 ± 0.3	4.1 ± 0.2

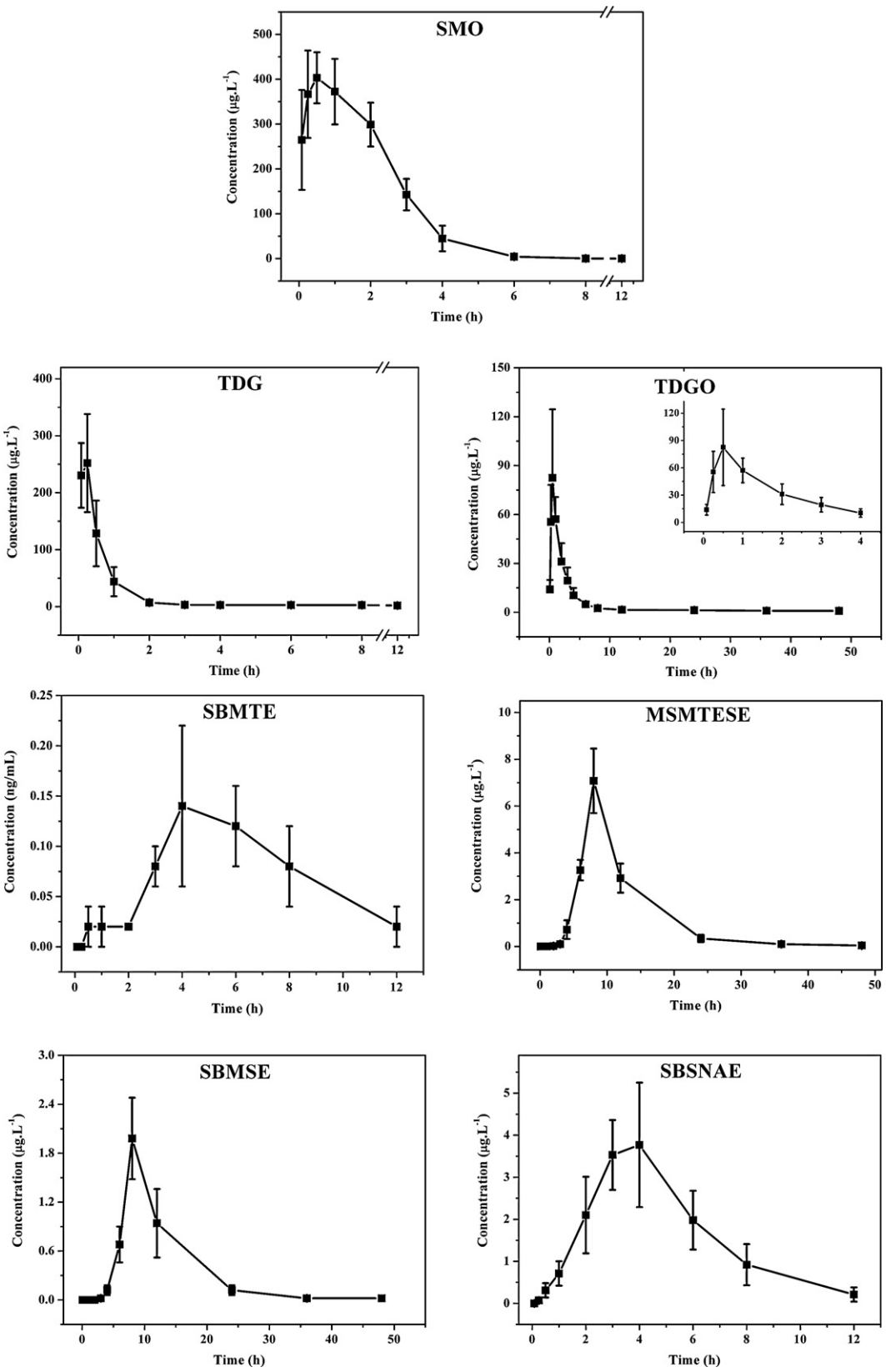

Fig. 3. Plasma concentration–time courses of the SM metabolites (TDG, TDGO, SMO, SBMTE, SBMSE, MSMTESE and SBSNAE) after a subcutaneous SM dose of 3.3 mg kg^{-1} in rats ($\mu \pm \sigma$, $n = 6$).

The maximum plasma concentrations (C_{max}) of 435.76 ± 48.64, 538.0 ± 167.8 and 82.52 ± 41.96 μg L^{-1} were measured in less than 1 h post exposure. However, a sharp decline of the plasma level from the peak was observed for these three biomarkers. Plasma levels dropped below the LLOQ after 4 h post exposure for TDG and 12 h for SMO and TDGO.

SMO was an oxidative metabolite of SM, which was initially reported by Davison et al. in 1961 [27] and was also identified by

Black et al. [11] to be a very minor metabolite in rat urine. Therefore, it was neither measured as a urine SM biomarker nor included in previously established methods. We found in this study that, unlike TDG and TDGO, no background level of SMO was detected in the blank plasma from either rats or human subjects who had no SM contact history (human data not show here). After SM exposure, SMO was a predominant component in the circulation of the rats, with a high plasma level in the time window of 12 h. This suggested that SMO can be used as an ideal plasma biomarker for SM poisoning, particularly in the first 12 h (latency period of SM poisoning) of a suspected exposure.

TDG and TDGO have been extensively studied and generally considered as SM biomarkers, particularly in urine samples. However, they are not regarded as unequivocal biomarkers because these two metabolites naturally occur at trace level in plasma and urine of either human or experimental animals [8,21]. They were detected in the previous studies as useful biomarkers to support the detection of β-lyase metabolites, which are regarded as definitive and unequivocal biomarkers of SM. In spite of this, much higher levels of TDG and TDGO were detected in the plasma of SM exposed rats, and the time windows for quantitative detection were 4 and 12 h respectively. They would provide an additional evidence for confirmation of SM poisoning. However, due to the existing background of TDG and TDGO in plasma, the actual plasma level below 10 $\mu g\,L^{-1}$ for TDG or 1 $\mu g\,L^{-1}$ for TDGO in a single point analysis should not be considered as a positive result for SM biomonitoring.

After subcutaneous injection of SM, the β-lyase metabolites were detected in the rat plasma collected at 1 h post exposure, and SBSNAE was quantitatively detected after 2 h. In this group, SBSNAE showed the highest plasma level, with the C_{max} at $8.56 \pm 2.54\ \mu g\,L^{-1}$. But the time window for quantification was only within 2–8 h post exposure, due to its relatively higher LLOQ (1 $\mu g\,L^{-1}$). MSMTESE and SBMSE could be detected and quantitative analyzed until 48 h after SM injection, with the C_{max} at 7.08 ± 1.38 and $1.98 \pm 0.50\ \mu g\,L^{-1}$, respectively. The mean retention times (MRT) in rat plasma for these two β-lyase metabolites were more than 11 h. MSMTESE and SBMSE have been viewed previously as unequivocal biomarkers of SM exposure [8]. With the additional advantages of the lower LLOQ and the relatively wide detection time window in plasma, they can also be used as plasma biomarkers. SBMTE, another β-lyase metabolite, was found to be a minor plasma metabolite of SM. It could be determined in the rat plasma from 1 to 8 h after exposure, but at a relatively lower level. The C_{max} was measured at $0.12 \pm 0.04\ \mu g\,L^{-1}$ only and the concentrations at the most sampling points were near or below the LLOQ. Therefore, no toxicokinetic parameters were calculated for SBMTE.

4. Conclusions

A simple and sensitive UPLC–MS/MS method was established for quantitative analysis of seven SM plasma metabolites in the study. The protein precipitation based one-step procedure allowed a rapid sample preparation, which also formed a good base for upgrading it to an automated high through-put procedure with 96-well plates. The method was optimized for simultaneous quantification of seven biomarkers in 13 min and the LLOQ was in the range of 0.01–1 $\mu g\,L^{-1}$ for most of the analytes, and at 5 $\mu g\,L^{-1}$ for TDG. The method was fully validated to perform quantitative analysis. The plasma profiles of the seven metabolites in SM exposed rats were explored by using this novel method. SMO, TDG, TDGO, SBSNAE, MSMTESE and SBMSE were quantitatively measured in the plasma of SM exposed rats at the levels well above the LLOQ. The reasonably wide time windows for SM biomonitoring were also observed. The method provides a useful tool for diagnosis of SM poisoning at the early stage of a suspected exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.12.035.

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