Validation of an HPLC–MS–MS Method for the Determination of Urinary S-Benzylmercapturic Acid and S-Phenylmercapturic Acid

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Abstract

A high-performance liquid chromatography–tandem mass spectrometric (HPLC–MS–MS) method is presented and evaluated for the determination of *S*-benzylmercapturic acid (*S*-BMA) and *S*-phenylmercapturic acid (*S*-PMA) in human urine. Both of these compounds are important for occupational health owing to their use as biomarkers of exposure to toluene and benzene, respectively. Toluene is used extensively as a solvent, and the health hazards of benzene have been well established. The optimized urine sample preparation scheme consists of solid-phase extraction (SPE) followed by an acetone wash. The chromatographic analysis consists of a reversed-phase gradient system, which uses electrospray ionization in negative-ion mode with a triple-quadrupole mass spectrometric detector. Accuracy and precision of this method are demonstrated by a series of recovery studies of spiked human urine and synthetic urine substitute. Spike levels at 1, 2, 6, 8, and 30 ng/mL for both analytes demonstrate average recoveries (accuracy) ranging from 99 to 110%. Precision as measured by the relative standard deviation (%RSD) of multiple samples (n = 9) at each concentration level was 5.3% or less for both analytes in urine. The limit of detection (LOD) is approximately 0.2 ng/mL for *S*-BMA and *S*-PMA. This data, other figures of merit and other factors, such as ion suppression of the electrospray ionization source, are discussed.

Introduction

Aromatic hydrocarbons such as benzene and toluene are of research interest with respect to human exposure and their toxicological and health effects. Toluene is used extensively in our modern industrial society owing to its properties as a solvent. It is a component in many consumer products, including beauty products and paints. Toluene has a multitude of industrial uses including the synthesis of other chemicals as well as being a common degreasing agent (1,2). Toluene is also a common component in gasoline and jet fuels (3). NATO military usage of toluene containing jet fuel alone is in excess of five billion gallons each year (3), and civilian usage of toluene containing fuels is much greater. Our modern motor vehicle-based society represents one of the main emission sources for toluene; thus, the general population undergoes lifelong exposure. Due to this extensive environmental exposure to toluene, the selection of an appropriate biomarker of exposure has been of considerable research emphasis in recent years (4) and is of interest to this laboratory. Benzene is classified as a group 1 human carcinogen by the International Agency for Research on Cancer (5). Although the use of benzene as a general solvent has been reduced significantly, it can still be found as a component in certain fuels as well as having use in the synthesis of other chemicals. Therefore, human exposure is not uncommon today. Furthermore, benzene can be released by several combustion reactions and has been found in minute quantities in tobacco smoke (6,7), and elevated urinary levels of benzene metabolites have been detected in smokers (8,9). The identification and the use of various biomarkers of benzene exposure is actively researched in the literature (8,10–16).

The metabolism of benzene and toluene have been thoroughly studied, and the applicability of their metabolites as possible biomarkers of exposure in humans has been discussed in the literature (15,17,18). Some of the common urinary metabolites of benzene include: *S*-PMA, *trans*, *trans*-muconic acid (*t,t*-MA), hydroquinone, catechol, phenol, and trihydrobenzene (17,19). Phenol and its conjugates are the main biotransformation products for benzene; however, these compounds are not suitable as specific biomarkers for benzene because they can be the products of other chemicals. Because a desired quality of a biomarker of exposure is that it should be specific for the chemical of interest, *S*-PMA is the preferred metabolite (20). Some of the known common urinary metabolites of toluene include *S*-BMA, hippuric acid, and phenylglyoxylic acid (12). Similarly, the corresponding mercapturic acid of toluene, *S*-BMA, is the preferred metabolite for use as a biomarker of exposure (4,21). Therefore, these mercapturic acid metabolites are the best choice for biomonitoring studies and were the target analytes for the current work.

* Disclaimers: Mention of company names and/or products does not constitute endorsement by the Centers for Disease Control and Prevention (CDC). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health (NIOSH).

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There have been numerous HPLC–MS analytical methods reported in the literature for the detection and quantification of either one or both *S*-PMA and *S*-BMA in urine (12,17,20,22). Sabatini et al. (22) reported the most completely validated method following the standards of the US Food and Drug Administration (FDA) (23) for the simultaneous determination of *S*-PMA, *S*-BMA, and *o*-methylbenzylmercapturic acid, a metabolite biomarker of xylene exposure. However, a method conforming to the validation standards of the National Institute for Occupational Safety and Health (NIOSH) as well as the generally accepted standards of method validation (24) was desired. A method which utilized a small-bore analytical HPLC column was required, as it could be more easily transferred between laboratories as opposed to the micro-bore HPLC system reported in the literature (22). In addition to the aim of having a method applicable to NIOSH instrumentation, a general improvement in and a more accurate determination of the detection limits over the methods reported in the literature was desired. Owing to the fact that most human urine contains some *S*-BMA from general environmental toluene exposure, Sabatini et al. (22) were not able to accurately define a limit of detection (LOD) during their study. Human sources of urine cannot provide a zero level or near zero level of *S*-BMA. UriSub, a synthetic urine substitute, was evaluated in the current work in an effort to more accurately determine an *S*-BMA LOD for the NIOSH method. Several alternative extraction procedures were also evaluated during the development of this method. The aim of the NIOSH method was to focus only on the appropriate biomarkers of toluene and benzene exposure; thus, *S*-BMA and *S*-PMA were the only target analytes. Finally, a small study of urine obtained from twelve volunteers was performed to verify that the method could be readily applied and give accurate results. Spiked and non-spiked samples were made from urine samples collected from both smokers and non-smokers.

Experimental

Instrumentation and chromatographic conditions

The chromatographic analysis was carried out using an Agilent Technologies model 1100 liquid chromatograph (Palo Alto, CA) with pump, degasser, and cooled autosampler, and it was equipped with an Agilent model 6410A triple quadrupole mass spectrometer (MS–MS) with an electrospray ionization (ESI) interface. The general chromatographic conditions are summarized in Table I. It should be noted that a post run of 100% mobile phase B at the higher 0.4 mL/min flow rate for 8 min was included to remove various well-retained urine sample components from the column before the system was re-equilibrated back to initial conditions. The MS–MS was run in multiple reaction mode (MRM) and the conditions used are also summarized in Table I.

Chemicals and reagents

The *S*-benzylmercapturic acid (*S*-BMA, *N*-acetyl-*S*-benzyl-*DL*-cysteine, CAS no. 19542-77-9) reference standard was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). The *S*-phenylmercapturic acid (*S*-PMA, *N*-acetyl-S-phenyl-*DL*-cysteine, CAS no. 20640-68-0) reference standard was purchased from Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan). The deuterated analogs, *S*-benzyl-d_5-mercapturic acid and *S*-phenyl-d_5-mercapturic acid were purchased from CDN Isotopes (Quebec, Canada). Acetonitrile and HPLC-grade water were obtained from Burdick & Jackson (Honeywell International, Inc., Muskegon, MI). Water for all sample preparation was obtained from a Barnstead NANOpure system (Thermo Fisher Scientific, Pittsburgh, PA). UriSub, the synthetic urine substitute, was purchased from CST Technologies, Inc. (Great Neck, NY). All other reagents used were of analytical grade and are regularly available in a laboratory.

General urine and UriSub sample preparation

Non-fortified urine or UriSub samples or analyte fortified samples were treated identically. Each was prepared by accurately measuring 4.0 mL of the urine or UriSub urine substitute into glass tubes and adding a 0.5-mL aliquot of a 30 ng/mL deuterated *S*-BMA and *S*-PMA internal standard solution. This made the

Table I. Instrumental Parameters of the Method

HPLC Conditions:

HPLC: Agilent Model 1100 pump and autoinjector (8°C sample vial cooling).
Column: Agilent Zorbax Rx C18, 3.5 µm, 250 × 3.0 (i.d.) mm
Guard Column: Pheomenex C18 SecurityGuard 4 × 2 mm
Column Flow: 0.3 mL/min [except for post run]
Injection size: 8 µL
Data Acquisition Time: 20 min
Mobile Phase:
A = 5:95:0.1% acetonitrile–water–acetic acid
B = 75:25:0.1% acetonitrile–water–acetic acid

Gradient Program

Run Time (min)	Mobile Phase Composition	Comments
0–10	0–40 % B	First gradient
10–18	40–100 % B	Second gradient
18–20	100 % B	End
20–28	100 % B (Flow to 0.4 mL/min)	Post Run

MS–MS Conditions:

Mass Spectrometer (MS–MS): Agilent Model 6410A with Electrospray Ionization Source in negative ion mode
Electrospray Voltage: 3500 V
Nebulizer Gas Flow: 10 L/min
Nebulizer Gas Pressure: 35 psi
Nebulizer Gas Temperature: 325°C
Fragmentor Voltage: 80 V for all analytes
Collision Gas: Nitrogen
Collision Gas Flow Rate: 0.06 L/min (factory default)
Collision Energy: 8 V for all analytes
Dwell Time: 200 ms
Mass Transitions: *m/z* 252 to 123 for S-BMA, *m/z* 238 to 109 for S-PMA, *m/z* 257 to 128 for deuterated S-BMA, and *m/z* 243 to 114 for deuterated S-PMA

internal standards equivalent to a 3.75 ng/mL level for the original 4-mL volume of urine. A 0.5-mL portion of deionized water for testing of the sample or analyte spiking solution was added to the fortified samples. For the primary recovery study, fortified samples were prepared equivalent to 1, 2, 8, and 30 ng/mL *S*-BMA and *S*-PMA levels in urine. Nine samples at each level were prepared and chromatographed (three at each level during each analytical batch run). A secondary recovery study using individual urine samples were fortified at an equivalent 6 ng/mL level of the two analytes. Urine samples were stored in a standard freezer and thawed before preparation.

Extraction

The optimized solid-phase extraction (SPE) procedure utilized Bond Elut C18 cartridges (Varian, Inc., Harbor City, CA) containing 500 mg of solid phase bed. The entire 5-mL volume of the prepared urine or urine substitute samples were passed through a prewashed SPE cartridge by means of a vacuum, followed by a 1.0 mL wash of water and collection of the extract by three 3 mL aliquots of acetone. The combined acetone solutions were evaporated by means of a LABCONCO CentriVap Concentrator (Kansas City, MO). The dry extracts were dissolved in 1.0 mL of mobile phase A before chromatographic analysis. Alternative extraction procedures were also evaluated during this study including the use of liquid–liquid extraction (LLE) by means of ethyl acetate and other SPE procedures utilizing Bond Elut C8 and Isolute ENV+ (Biotage, Uppsala, Sweden) cartridges using acetonitrile, acetonitrile–water mixtures, and acetone as extraction solvent using the sample general procedure.

Standard sample preparation

Calibration standard samples were prepared at 0, 0.5, 1, 2, 5, 10, 20, 40, and 50 ng/mL urine equivalent levels of *S*-BMA and *S*-PMA and a fixed level (3.75 ng/mL equivalent level) of the two internal standards in a solution having the same composition as mobile phase A.

Calculations

All analyte quantification for the validation of this test method was based on peak area ratios of the *S*-BMA and *S*-PMA analytes to their respective deuterated analogs. The initial extraction efficiency studies and ion suppression studies were based on peak areas of the analytes compared to chromatograms of known concentration standard solutions. The standard calibration curves used for the recovery studies were linear within the 0.5 to 50 ng/mL range used; correlation coefficients were 0.98 or greater and y-intercepts approached zero for all curves generated with this chromatographic procedure. Calibration curves were generated at the beginning, middle, and end of each recovery chromatographic run to verify the lack of any significant calibration curve drift during the use of this method. Sample quantification was calculated using the bracketed standard curves, the preceding and following curves were used and the two assay values were averaged. Multiple calibrations curves were collected and analyzed during the validation process of this method.

The limit of detection (LOD) was calculated in the traditional manner (23,24), using three times the noise level of the chromatogram's baseline divided by the slope of a calibration curve. Because instrument noise is a function of peak height, the average baseline level of height noise was determined for each batch run in chromatograms at the retention time window for each analyte from non-fortified urine samples. It should be noted that ion suppression was taken into account for this determination, which was found to be a 30% reduction in peak response for urine and a 40% reduction in peak response for UriSub® [see Results and Discussion section on ion suppression measurements and considerations]. The slope from the calibration curve using peak heights of the standards was used as the divisor for this LOD calculation. It should be stressed that peak heights were used only for the estimation of the LOD of this method; peak area ratios were used for the quantification of the target analytes during the recovery studies of the method's validation.

Results and Discussion

Chromatography and detection

The optimized chromatographic conditions developed for this method proved to be specific and have no major interferences; these conditions enabled the simultaneous quantification of both *S*-BMA and *S*-PMA. Generally, tandem mass spectrometric detection gives a high degree of specificity to an HPLC method. The most critical element of a validation is in proving the specificity of the method, which was demonstrated by the analysis of various non-fortified urine samples from individuals during this study. All non-fortified urine samples showed no interfering peaks for *S*-PMA and the internal standards, although all human urine contained some *S*-BMA, which always appeared as one distinct peak in the chromatograms generated. A typical chromatogram is displayed in Figure 1A, which shows signal plots from a fortified urine sample containing 1 ng/mL *S*-PMA (retention time approximately 14.5 min, *m/z* 238 → 109) and 7.2 ng/mL *S*-BMA (retention time approximately 16 minutes, *m/z* 252 → 123). The deuterated internal standards (IS) plots are also displayed in Figure 1 (*m/z* 243 → 114, *m/z* 257 → 128). The chromatographic baselines displayed little drift from the gradient run and none of the background peaks interfered with the target analytes. It should be noted that the internal standard peaks were well resolved from any other peaks appearing in the chromatogram. This method also offers improvement from possible interferences from other chromatographic systems reported in the literature (22). The chromatogram displayed in Figure 1B shows non-fortified urine containing only background *S*-BMA (6.2 ng/mL), which confirms the lack of interferences for the deuterated internal standard plots and *S*-PMA. Sample carryover was eliminated in this method by the use of a 50/50 (v/v) acetonitrile–water rinse of the autosampler injector needle and loop. This also eliminated a problem with artifact peaks, which were noted during the early development stage of this method. Artifact peaks from human urine also required the use of a more extensive post-run column wash. It was found necessary to wash the column with 100% mobile phase B (75:25:0.1, acetonitrile–water–acetic acid, v/v/v) for a minimum of 8 min at a flow rate of 0.4 mL/min to completely remove artifact urine components from previous injections.

Ion suppression measurements and considerations

The amount of ion suppression of the analytes involved in this method was studied; suppression sources investigated included both those from the mobile phase composition and the urine and UriSub related sample matrices. The general factors that cause quenching or suppression of complete analyte ionization related to mobile phase composition and electrospray ionization (ESI) sources have been extensively discussed elsewhere (14,25,26). The 0.1% (v/v) acetic acid mobile phase used in this validated method was found to give better analyte response than the 20 nM formic acid based mobile phases suggested in the literature (12,22). An acetonitrile–water based mobile phase system made in 20 nM in formic acid was found to have reduced the response for both *S*-BMA and *S*-PMA; an approximate 40% response reduction was determined when compared to the acetic acid mobile phase system used in the final method.

The sample matrix was also found to generate some ion suppression, which was most likely caused by co-eluting components competing for ionization with the analytes. This process and possibility have been generally described in the literature (14,25,27). Urine extracts were spiked with known levels of *S*-BMA and *S*-PMA, chromatographed, and detector responses were compared to known standard solutions of the analytes prepared in straight mobile phase A. This is the sample matrix suppression experimental procedure recommended by the U.S. Food and Drug Administration for bioanalytical method validation (23). Biological fluids tend to be very complex mixtures and inter-subject variability of urine, in particular, can cause various problems. Although the detector response of target analytes was found to vary with the individual urine source, generally response was reduced by approximately 30% by ion suppression in the optimized acetic acid HPLC system. This was also using the 8 µL injection volume and the 1.0 mL dissolution volume of the dry urine extract. The substitute urine, UriSub, was found to increase ion suppression to a greater extent; detector response was reduced by approximately 40% and was more significant for *S*-PMA than *S*-BMA. The injection volume and concentration of the urine extracts were evaluated during the method's development and were optimized for analyte response and ease of sample preparation. Injection volumes of 5, 8, and 10 µL were investigated at various concentrations of urine extract. Higher extract concentrations or larger injection volumes increased ion suppression to a point where only minimal increases in analyte detector response were obtained. The 1.0 mL dissolution volume made for a more complete transfer to an autosampler vial. The 8-µL injection volume and 1.0 mL dissolution volume of the dry extract were found to give the best procedural compromise, so these parameters were used for the final method.

Mass spectra of the analytes

The mass spectrometric analysis of *S*-BMA and *S*-PMA have been described in the literature (20,22). Figure 2 shows the product ion mass spectra of the two analytes which were obtained in negative ion mode from the precursor molecular ions. The mass transitions monitored for quantification of the analytes were *m/z* 252 → 123 for *S*-BMA and *m/z* 238 → 109 for

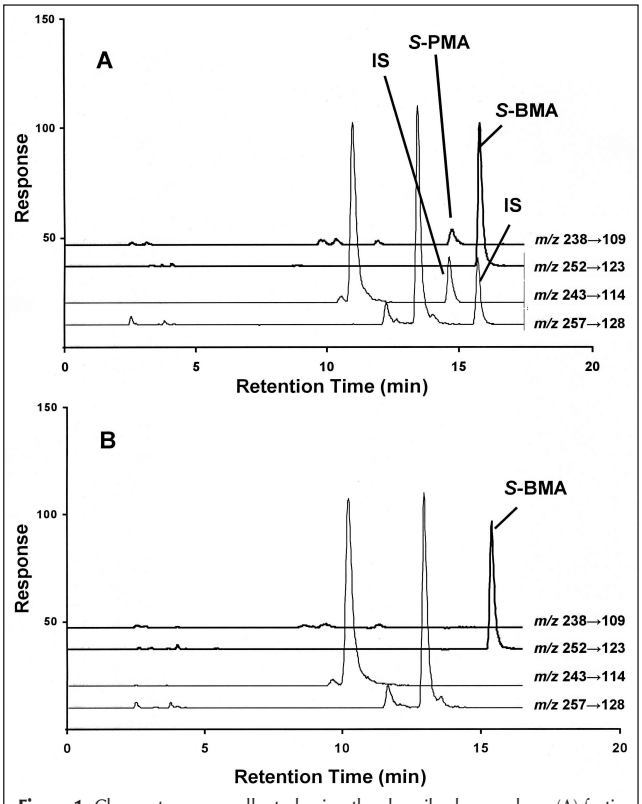

Figure 1. Chromatograms collected using the described procedure: (A) fortified urine sample containing 1 ng/mL S-PMA, 7.2 ng/mL S-BMA and 3.8 ng/mL of the corresponding deuterated internal standards; (B) non-fortified urine from above containing only the 6.2 ng/mL background level of S-BMA.

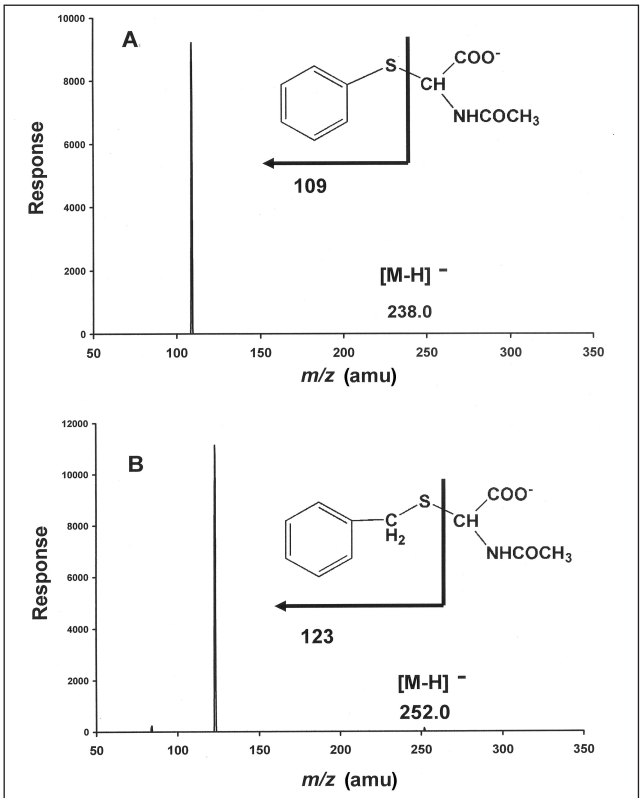

Figure 2. Product mass spectra using ESI in negative mode and ion structures of S-PMA, precursor ion *m/z* = 238 and S-BMA, precursor ion *m/z* = 252, using the mass spectral conditions of the test method. The fragmentor voltage was 80 V and the collision energy was set at 8 V for both molecular ions.

S-PMA which was the major ion products. This was consistent with previously reported results (20,22). The main fragments detected for these two compounds resulted from the deprotonated molecular ions by loss of CO_2 and CH_2=CHNHCOCH$_3$. The corresponding transitions of *m/z* 257 → 128 (d_5 *S*-BMA) and *m/z* 243 → 114 (d_5 *S*-PMA) were monitored for the internal standards.

Extraction conditions development

The use of the Bond Elut C18 SPE and acetone for analyte elution proved to have the highest extraction efficiency; *S*-BMA and *S*-PMA had recoveries of 74 and 66% (n = 3) respectively. Acetone for use in elution with SPE has been successfully used for the analysis of other urinary metabolites and reported in the literature (28). Using the C18 SPE cartridges and 85/15 (v/v) acetonitrile–water for elution had slightly lower recovery; 72 (*S*-BMA) and 60% (*S*-PMA, n = 3) for the analytes. Pure acetonitrile yielded less of the analytes. Bond Elut C8 demonstrated recoveries ~ 10% lower than the C18 cartridges. Owing to the higher aqueous solubility and partitioning of the mercapturic acid metabolite, liquid–liquid extraction (LLE) using ethyl acetate proved to be the least efficient; recoveries of both metabolites from urine were 52 (*S*-BMA) and 58% (*S*-PMA, n = 3), respectively. The Isolute ENV+ proved to be generally incompatible with any elution solvent tried and gave low yields of the analytes. Because this method used procedural internal standards, deuterated analogs for both target analytes, the extraction efficiency of the Bond Elut C18 was more than adequate for this biomarker assay method.

Linearity, choice of standard preparation, and LOD

The linearity of the method was determined over the range of 0.5 to 50 ng/mL for both *S*-BMA and *S*-PMA. The use of isotopically labeled analogs is typically ideal for HPLC-ESI-MS and was used for this method. Each calibration equation was fitted by linear regression where the peak area ratio between the analyte and its respective internal standard were used. For both analytes, the calibration curves generated showed correlation coefficients (r^2) of 0.98 or greater. Various procedures for the preparation of the standard solutions were tried during early method development. Preparation of standard solutions from both reference urine and UriSub were evaluated. The slope of the calibration curves generated between urine and UriSub extracts and solutions prepared at equivalent levels in the same composition as mobile phase A were not statistically different. Since the simple preparation of equivalent levels of standards in mobile phase A would lead to higher laboratory through-put, this was the standard procedure adopted for the validated method. Also, preliminary recovery data obtained further demonstrated equivalency between the standard preparation procedures.

The limit of detection (LOD) was determined to be approximately 0.2 ng/mL for *S*-PMA in either urine or UriSub extract sample matrices. The limit of detection (LOD) was calculated in the traditional manner (23,24) using three times the noise level of the chromatogram's baseline divided by the slope of a calibration curve using peak heights and accounting for sample matrix ion suppression. The determination of an accurate LOD for *S*-BMA from urine extract sample was not practical owing to the background levels of the metabolite in all human urine. This problem has been reported previously in the literature (22). The LOD of *S*-BMA extracted from UriSub was estimated to be approximately 0.2 ng/mL. The similarity of urine and UriSub extracts, plus the determination of the LOD of the chemically similar metabolite *S*-PMA, is adequate to predict an approximate LOD for *S*-BMA of approximately 0.2 ng/mL for this method. The limit of quantitation (LOQ) can be expected to be approximately 0.7 ng/mL by definition; that is 3.3 times the LOD.

Precision and accuracy of the optimized conditions

A primary recovery experiment using the optimized conditions with reference urine fortified with *S*-BMP and *S*-PMA was performed to demonstrate the accuracy and precision of the method. The reference urine in this study was found to contain a background level of 6.2 ng/mL *S*-BMA and no detectable quantity of *S*-PMA. These data are presented in Table II; average recovery ranged from 103 to 106% for the four fortified levels of *S*-BMA investigated and from 102 to 109% for the four levels of *S*-PMA (percent recovery of theory is based upon the known levels of standards added to the urine samples). For each analytical batch run, the experimental trial consisted of three urine samples prepared at four concentration levels. Because it was not possible to obtain human urine without background *S*-BMA, UriSub was evaluated by the same type of recovery experiment to demonstrate that the method could accurately determine low levels of that metabolite. These data are presented in Table III; mean recovery ranged from 102% to 107% for the four fortified levels of *S*-BMA investigated and from 99 to 109% for the four levels of *S*-PMA. The recovery results have mean values of accuracy, which are acceptable for a bioanalytical method; the means are within the required plus or minus 15% of the theoretical values (23). There may be some high bias to this method, but it is within acceptable limits. Since the method utilizes deuterated analogs of the analytes as internal standards, any high bias is unlikely to be from any ion enhancement characteristics of the electrospray

Table II. Multiple Level Urine Recovery Experiment of *S*-Benzylmercapturic Acid and *S*-Phenylmercapturic Acid

Analyte conc. added (ng/mL)	Mean measured conc. (n = 9) (ng/mL)	Mean percent recovery (Accuracy)	Standard deviation (ng/mL)	%RSD*
S-BMA†				
1	7.4	103	0.37	5.0
2	8.4	103	0.19	2.3
8	14.6	103	0.32	2.2
30	38.2	106	0.94	2.4
S-PMA				
1	1.0	102	0.05	5.3
2	2.1	105	0.09	2.3
8	8.2	103	0.27	2.3
30	31.9	109	0.65	2.0

* Note: %RSD is percent relative deviation.
† The non-fortified reference urine had a background level of 6.2 ng/mL S-BMA and no detectable level of S-PMA. Recoveries are based on the background level plus the added metabolite.

source. The precision of the method is also acceptable for the urine extracts. The UriSub had much less precision, especially at the low concentration levels. This was most likely due to the much higher ion suppression, thus lower detector response, of the UriSub extracts. It can be concluded that UriSub was not a good substitute for urine with respect to assay precision using this method.

A second recovery experiment was performed on urine collected from smokers and non-smokers; 6 ng/mL level spiked urine samples were used. These data are presented in Table IV and recovery is calculated as a percentage of the background level of the metabolites plus the 6 ng/mL spike. Mean recovery for smokers was 99% for *S*-BMA and 110% for *S*-PMA (n = 6). Mean recovery for non-smokers was 102% for *S*-BMA and 109% for *S*-PMA (n = 6). The background measurements showed mean levels of 10.5 ng/mL *S*-BMA and 0.4 ng/mL *S*-PMA for smokers (n = 6), and 8.2 ng/mL *S*-BMA; *S*-PMA was not detected for non-smokers (n = 6). This small group of individuals was too small to draw any conclusion, but it did demonstrate the applicability of the method.

Method reproducibility, analyte stability, and other considerations

Two different Agilent Zorbax RX C18 columns of different batch numbers were used during the recovery experiments; therefore, the method's quantatitive results and chromatographic performance are expected to be consistent and reproducible with other columns produced by the manufacturer. This would also indicate the method's robustness for the chromatographic system used. A six day stability experiment was conducted using the final chromatographic sample solution. Conditions included room temperature and 8°C with minimal light exposure by means of flint glass sample vials and exposure to sunlight/ambient laboratory light at room temperature using clear glass sample vials. Both *S*-BMA and *S*-PMA were demonstrated to be stable when stored with minimal light exposure for six days, either at room temperature or at 8°C. After 24 hours of storage in light at room temperature, *S*-BMA and *S*-PMA had mean assay values of 75 and 72% (n = 3), respectively, when compared to solutions of freshly prepared reference standards. After three days of light exposure, extensive degradation was noticed; *S*-BMA mean assay values had fallen to 9% (n = 3) of the original level and *S*-PMA had degraded to 16% (n = 3). After six days of exposure to light, both analytes were nearly completely degraded. Even though both analytes benefit from the use of individual deuterated internal standards; the use of flint glass autosampler vials or other means of reducing light exposure is recommended when using this method to insure a high detector response for extended chromatographic batch runs.

Table III. Multiple Level UriSub Recovery Experiment of S-Benzylmercapturic Acid and S-Phenylmercapturic Acid

Analyte conc. added (ng/mL)	Mean measured conc. (n = 9) (ng/mL)	Mean percent recovery (Accuracy)	Standard deviation (ng/mL)	%RSD*
S-BMA				
1	1.1	107	0.31	29.0
2	2.1	106	0.34	16.0
8	8.3	104	0.54	6.4
30	30.7	102	0.76	2.5
S-PMA				
1	1.1	109	0.27	25.0
2	2.0	99	0.16	7.9
8	8.0	100	0.48	6.0
30	31.1	104	0.91	2.9

* Note: %RSD is percent relative standard deviation

Future Work

Future work in this laboratory will include the application of this validated method for various workers exposed to either one or both toluene and benzene, which is beyond the scope of this report. This developed method, along with other biomarker methods developed at this laboratory (29,30) will be applied to fuel-tank-maintenance workers within the United States Air Force. Also, many manicurist supplies and beauty aids are known to be formulated with toluene and an exposure study of manicurists is planned as future work. These are part of larger and more comprehensive studies and are beyond the scope of this reported work. The focus of this work is the description and validation of the HPLC–MS–MS assay method.

Table IV. Individual Smoker and Non-Smoker Recovery Experiment of S-Benzylmercapturic Acid and S-Phenylmercapturic Acid*

	Background Level		6 ng/mL Fortified Sample Recovery	
Individual Sample	S-BMA (ng/mL)	S-PMA (ng/mL)	S-BMA [ng/mL (%)]	S-PMA [ng/mL (%)]
Smoker				
1	2.7	0.2	8.6 (99%)	6.5 (104%)
2	28.3	0.3	34.7 (101%)	6.9 (108%)
3	15.9	0.9	31.3 (97%)	7.9 (114%)
4	5.7	0.3	11.6 (99%)	7.7 (121%)
5	1.3	n.d.†	6.9 (95%)	6.4 (106%)
6	9.2	0.7	15.5 (102%)	7.0 (104%)
Mean =	10.5	0.4	16.4 (99%)	7.1 (110%)
Non-smoker				
1	0.3	n.d.	6.2 (98%)	6.2 (103%)
2	7.1	n.d.	14.3 (109%)	7.4 (123%)
3	6.8	n.d.	13.1 (102%)	6.8 (112%)
4	23.3	n.d.	28.6 (97%)	6.3 (105%)
5	4.7	n.d.	11.0 (103%)	6.3 (105%)
6	7.2	n.d.	13.5 (102%)	6.1 (101%)
Mean =	8.2	–	14.5 (102%)	6.5 (109%)

* Notes: The limit of detection (LOD) was estimated at 0.2 ng/mL for both analytes. Values between the LOD and 1.0 ng/mL were reported as one significant figure.
† n.d. = none detected or less than LOD.

Conclusions

An analytical method to measure the levels of the biomarkers *S*-BMA and *S*-PMA in urine samples was developed and fully validated. Solid-phase extraction of urine sample by means of a C18 cartridge and elution of the analytes by acetone was the most effective procedure. The method was demonstrated to be accurate and precise within the definitions of the United States FDA. The LOD of the method was estimated to be approximately 0.2 ng/mL for both analytes and linearity was established at a urinary concentration of 0.5 to 50 ng/mL. The method was demonstrated to be applicable to collected human urine samples.

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