BACKUP DATA REPORT
Method No. 8326
_S_-Benzylmercapturic acid and _S_-Phenylmercapturic acid in urine
Metabolites of toluene and benzene
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Substance(s): 1. _S_-Benzylmercapturic acid, BMA
2. _S_-Phenylmercapturic acid, PMA
Exposure Limits: Not applicable

Chemicals Used for Evaluation:
1. _S_-Benzylmercapturic acid (_N_-acetyl-_S_-benzyl-DL-cysteine) from Cambridge Isotope Laboratories, Inc., (Tewksbury, MA, USA) 98%, Lot no. I1-7133.
2. _S_-Phenylmercapturic acid from Tokyo Kasei Kogyo, Ltd., 98%, Lot no. FIH01.
3. DL-_S_-Benzyl-d_5-mercapturic acid (d_5-benzylmercapturic acid, d_5-BMA) from CDN isotopes, Inc., (Pointe-Claire, Quebec, Canada) 98%, Lot no. P231P1.
4. DL-_S_-Phenyl-d_5-mercapturic acid (d_5-phenylmercapturic acid, d_5-PMA) from CDN isotopes, Inc., 98%, Lot no. P231P1.

GENERAL

Synopsis

_S_-Benzylmercapturic acid (BMA) is a metabolite of toluene and is a proposed biomarker of exposure for that chemical. _S_-Phenylmercapturic acid (PMA) is a metabolite of benzene and is a biomarker of exposure for that chemical. Both parent chemicals are common solvents and components in many petroleum-based products and mixtures; thus, exposure to one or both chemicals is not uncommon for many workers in a variety of occupations. The described procedure was developed for the detection and quantification of the two metabolites in urine. Urine from an exposed population is collected, shipped cold and stored frozen at -70 to -80 °C. The samples are thawed for analysis. Solid-phase extraction (SPE) using C18 cartridges is used to collect the two analytes from the urine samples. The acetone extracts from the SPE cartridges are evaporated and the dry residues are dissolved in mobile phase A (5/95/0.1% (v/v/v) acetonitrile/water/acetic acid) before high-performance liquid chromatographic (HPLC) analysis. Detection is by means of a tandem mass spectrometer (MS/MS). The deuterated analogs of both analytes, d_5-BMA and d_5-PMA are used as internal standards.

Applicability

PMA is a very specific biomarker for benzene. BMA can form from exposure to other sources, such as benzyl acetate or benzyl alcohol; which can be found in personal care products [1]. Both toluene and benzene are common solvents with multiple occupational uses; furthermore, exposure to toluene and benzene (less commonly) can occur from environmental and other sources. This method measures the quantity of the two target metabolite analytes in urine. Caution must be used as background levels of PMA and BMA can be found in urine due to non-occupational (environmental and recreational) exposures, especially smoking. This method will accurately determine total exposure from all sources and all routes (dermal, inhalation, ingestion) to toluene and benzene by measuring these two metabolites. Specific sources of exposure cannot be differentiated using this method.

Method Evaluation

This method was evaluated in accordance with the general guidance documents for typical method validation (References 2 and 3). Furthermore, this method has been described by B'Hymer (References 4 and 5). The key elements of method validation including accuracy, precision, linearity, specificity, robustness, stability and limit of detection have been investigated during this method's development. The accuracy and precision were determined by spiked urine sample recoveries studied at four different concentration levels and are described in detail later in this report. The other elements are also described in detail with their respective results within this report.

Sampling Aspects

All urine samples for testing were prepared by mixing 4.0 mL of urine with 0.5 mL of water. In the case of spiked urine samples, they alternatively were prepared by adding 0.5 mL of the appropriate aqueous spike level solution. All samples were spiked with a 0.5 mL aliquot of a 30 ng/mL deuterated BMA/PMA solution, which are the internal standards. Solids in urine samples caused no problems during the solid phase extraction; therefore, filtering the urine was not included in this test method's sample preparation procedure.

Analytical Aspects

The chromatographic analysis was carried out using an Agilent Technologies model 1100 HPLC pumping system with autosampler (Palo Alto, California, USA) and an Agilent Technologies model 6410A triple quadrupole mass spectrometer used as the detector. The detector output was processed by Agilent's Mass Hunter software where all data were evaluated and the chromatographic peaks were integrated. The column used was an Agilent Zorbax Rx-C18 (150 X 3 mm, 3.5 µm particle size) using gradient elution with acetonitrile/water mobile phases containing 0.1% acetic acid. The chromatographic and mass spectrometric conditions were optimized for this method and are described below:

Chromatographic Conditions

Mobile Phases: A = 5/95/0.1% (v/v/v) acetonitrile/water/acetic acid
B = 75/25/0.1% (v/v/v) acetonitrile/water/acetic acid

Flow Rate: 0.3 mL/min (during analysis time)

Gradient Program:	Time (min)	Mobile Phase Composition	Comments
	0 to 10	0 to 40% B	Initial gradient ramp
	10 to 18	40 to 100% B	Second gradient ramp
	18 to 20	100% B	Final hold
	20 to 21	100% B	Increase flow to 0.4 mL/min
	21 to 28	100% B	Column wash
	28 to 30	100 to 0% B	Decrease flow to 0.3 mL/min
	30 to 37	0% B	Column re-equilibration

Injection Volume: 8 µL

Mass Spectrometric Conditions

Ionization Source: Electrospray at 3500 Volts and negative scan mode, nebulizer gas at 35 psi and 10 L/min flow

Multiple Reaction Mode (MRM)**:**

Quantification mass transitions – BMA = *m/z* 252 → 123, PMA = 238 → 109, d_5-BMA = 257 → 128, d_5-PMA = 243 → 114.

The following table lists the instrumental settings used for the model 6410A mass spectrometer:

Analyte	Precursor Ion	MS1 Resolution	Product Ion	MS2 Resolution	Dwell Time (msec)	Fragmentor Voltage	Collision Energy (volt)
d_5-BMA	257	unit	128	unit	200	80	8
BMA	252	unit	123	unit	200	80	8
d_5-PMA	243	unit	114	unit	200	80	8
PMA	238	unit	109	unit	200	80	8

Extraction/Sample Preparation

Non-spiked urine samples and those spiked with the two analytes were treated identically. A 4.0 mL portion of the urine was placed in a screw-capped tube. A 0.5 mL aliquot of a 30 ng/mL d_5-BMA/d_5-PMA internal standard solution was added. A 0.5 mL portion of deionized water or the appropriate analyte spiking solution was added. The urine sample was extracted by means of a Varian Bond Elut C18 SPE cartridge. The SPE cartridge was pre-washed with 2 mL of acetone and re-equilibrated with 2 mL of water. The urine solution was passed through the cartridge by means of vacuum followed by a 1 mL water wash. The analytes were eluted by three 3-mL volumes of acetone passed through the SPE cartridge. The collected acetone extracts were removed and combined. The extract solutions were evaporated to dryness by means of a rotary concentrator at 30 °C. Prior to HPLC analysis, the dry extracts were dissolved in 1 mL of mobile phase A and transferred to an HPLC autosampler vial.

The chemical reagents used were those commonly found in a laboratory. The acetone, acetonitrile and methanol were HPLC grade. The sources of the reference compounds have been described previously.

Method developmental considerations

The use of the Bond Elut C18 SPE cartridges and acetone for analyte elution was chosen for this method due to the superior extraction efficiency of this combination for BMA and PMA. Recovery was found to be 74% (n=3) for BMA and 66% for PMA (n=3). Acetonitrile and acetonitrile/water mixtures gave lower recovery results. Bond Elut C8 SPE cartridges demonstrated recoveries about 10% lower than the C18 cartridges. Other extraction procedures were evaluated during the early development stage of this method. The Isolute ENV$^+$ SPE cartridge proved to be generally incompatible with any elution solvent tried and gave low yields of the target analytes. Owing to the high aqueous solubility and partitioning of the mercapturic acid metabolites, liquid-liquid extraction using ethyl acetate proved to be the least efficient; recoveries of both metabolites from urine were in the 50% range.

The standard calibration curve samples were prepared in separate solutions. During the method development, calibration samples were prepared by spiking a reference urine and by separate aqueous solutions prepared at equivalent levels. When compared to each other, the calibration curves had statistically identical slopes and produced recovery results with statistically identical values. It was decided, therefore, to not make the standard curve from fortified reference urine. This had the added benefit of making a less labor intensive method without sacrificing any accuracy.

The only other minor difficulty found during the development of this method was with minor sample carry-over by the autosampler. The use of a needle rinse with 50/50% (v/v) acetonitrile/water was found to be necessary to eliminate this problem.

RESULTS

Accuracy and Precision

Several recovery studies using two columns over several days demonstrated the accuracy and precision of this test method. A primary recovery experiment using the optimized conditions with reference urine fortified with BMA and 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 BMA and no detectable quantity of PMA. These data are presented in Table 1; average recovery ranged from 103 to 106% for the four fortified levels of BMA investigated and from 102 to 109% for the four levels of PMA. For each analytical batch run, the experimental trial consisted of three urine samples prepared at four concentration levels. Since it was not possible to obtain human urine without background BMA, a synthetic urine, UriSub (CST Technologies, Inc., Great Neck, NY, USA), 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 2; mean recovery ranged from 102 to 107% for the four fortified levels of BMA investigated and from 99 to 109% for the four levels of PMA. The recovery results have mean values of accuracy which are acceptable for a bioanalytical method; the means are within the required range of plus or minus 15% of the theoretical values (Reference 2). There may be some high bias to this method, but it is within acceptable limits. 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 3 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 BMA and 110% for PMA (n=6). Mean recovery for non-smokers was 102% for BMA and 109% for PMA (n=6). The background measurements showed mean levels of 10.5 ng/mL BMA and 0.4 ng/mL PMA for smokers (n=6), and 8.2 ng/mL BMA; PMA was not detected for non-smokers (n=6).

Linearity

All calibration curves used during the development of this method were found to be linear and had correlation coefficients of 0.99 and greater with y-intercepts close to zero. The procedure was found to be linear within the standard concentration ranges described; 0.5 to 50 ng/mL BMA and PMA equivalent levels in urine.

Specificity

The optimized chromatographic conditions developed for this procedure proved to be specific and have no major interferences, and enabled for the simultaneous quantification of the two target analytes. All non-fortified urine samples chromatographed showed no interfering peaks; the blank samples from 12 non-exposed volunteers showed no interferences for the internal standards or the target analytes at the selected mass transitions used for quantification. The chromatographic baselines displayed little drift from the gradient run and proved to be easily integrated for data analysis.

Robustness

Two Zorbax Rx-C18 columns were used during the recovery studies. Accuracy and precision did not appear to be affected; therefore, the method appears to be reproducible with any normal functioning Zorbax Rx-C18 HPLC column. Recovery results from individual urine samples spiked with the two analytes indicate that the method was accurate and not significantly affected by individual urine sample matrix differences during analyte extraction or chromatographic analysis.

Stability

Extract stability was evaluated. A six-day stability study was conducted on the final chromatographic sample solution. BMA and PMA appeared to be stable at 8°C (the autosampler temperature) and at room temperature in the absence of light. After 1 day of storage in light at room temperature, BMA and 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; BMA mean assay values had fallen to 9% (n=3) of the original level and PMA had degraded to 16% (n=3). After six days of exposure to light, both analytes were nearly completely degraded. Although both analytes benefit from the use of individual deuterated internal standards; the use of amber glass autosampler vials or other means of reducing light exposure is highly recommended when using this method to ensure sample stability during extended chromatographic batch runs.

Range

The procedure was found to be linear within the standard concentration ranges described: 0.5 to 50 ng/mL BMA and PMA equivalent levels in urine. Field samples at a higher level can be diluted to a concentration within that range for analysis.

Limit of detection

The limit of detection (LOD) was calculated in a traditional manner (References 1 and 2) using three times the noise level divided by the slope of calibration curves. Since instrumental noise is a function of 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 the blank samples. This was done by exporting raw data files into Microsoft Excel® and determining the mean height level and the standard deviation of height noise from 100 data points within the retention time window noted for specific analyte monitoring the specific transition signal. The slope from the calibration curve using peak heights of all the standard solutions was determined and then used as the divisor for this LOD calculation. It should be noted that peak height was used only for the estimation of the LOD; the peak area ratio was used for quantification of the analytes during the validation of this procedure. This “instrumental” LOD was found to be approximately 0.2 ng/mL for both BMA and PMA. It should be noted that the calibration curves used for this estimation were generated from fortified urine samples; therefore, ion suppression and recovery loss

was accounted for in this estimation of LOD. Also, it should be noted that the actual LOD is dependent upon the actual performance of the chromatographic system and the detector at the time of an actual analysis. Since this method calls for the lowest standard concentrations of 0.5 ng/mL for BMA and PMA, this can be considered the "operational" LOD and a basic criteria for the use of this method. If a column, chromatographic system or detector cannot detect the lowest standard level, corrective action would be required.

Ruggedness

Laboratory-to-laboratory reproducibility was not evaluated for this method at the time of this report. This method was originally developed for support of healthcare worker studies within the Biomonitoring and Health Assessment Branch (BHAB), Division of Applied Research and Technology (DART) at NIOSH and was not expected to be transferred. Reproducibility within the laboratory over the period of method validation was only performed and these data are shown in Tables 1, 2 and 3.

User Check data was generated in January 2013 for this method. Twenty-five samples (five blanks and five at each of four concentrations) were prepared and sent to a contract laboratory. Recoveries for BMA averaged 98.7% with an average relative standard deviation of 7.4%. Recoveries for PMA averaged 93.1% with an average relative standard deviation of 11.3%. These values are well within acceptable analytical criteria. A Summary table of the User Check data is shown below.

Spiked amount (ng/mL)	Compound	Recovery (%)	RSD (%)
2.46	BMA	98.7	6.3
2.49	PMA	81.1	4.9
4.93	BMA	98.4	9.9
4.98	PMA	90.0	5.0
14.78	BMA	98.6	8.2
14.93	PMA	95.1	8.2
34.49	BMA	99.0	4.1
34.83	PMA	106.2	3.8
Overall	BMA	98.7	7.4
Overall	PMA	93.1	11.3

Table 1

Multiple Level Urine Recovery Experiment of BMA and PMA

Analyte conc. added (ng/mL)	Mean measured conc. (n=9) (ng/mL)	Mean percent recovery	Standard deviation (ng/mL)	%RSD
BMA[1]				
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
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

1. The non-fortified reference urine had a background level of 6.2 ng/mL BMA and no detectable level of PMA. Recoveries are based on the background level plus the added metabolite.

Note: %RSD is percent relative deviation.

Table 2

Multiple Level UriSub® Recovery Experiment of BMA and PMA

Analyte conc. added (ng/mL)	Mean measured conc. (n=9) (ng/mL)	Mean percent recovery	Standard deviation (ng/mL)	%RSD
BMA				
1	1.1	107	0.31	29
2	2.1	106	0.34	16
8	8.3	104	0.54	6.4
30	30.7	102	0.76	2.5
PMA				
1	1.1	109	0.27	25
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

Table 3

Individual Smoker and Non-Smoker Recovery Experiment of BMA and PMA

Individual Sample	Background Level		6 ng/mL Fortified Sample Recovery	
	BMA (ng/mL)	PMA (ng/mL)	BMA [ng/mL (percent)]	PMA [ng/mL (percent)]
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	nd	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	nd	6.2 (98%)	6.2 (103%)
2	7.1	nd	14.3 (109%)	7.4 (123%)
3	6.8	nd	13.1 (102%)	6.8 (112%)
4	23.3	nd	28.6 (97%)	6.3 (105%)
5	4.7	nd	11.0 (103%)	6.3 (105%)
6	7.2	nd	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 the limit of quantitation (LOQ) were reported as one significant figure with obvious limitations. nd = none detected or less than LOD.

References

[1] Lovreglio P, Barbieri A, Carrieri M, Sabatini L, Fracasso ME, Doria D, Drago I, Basso A, D'Errico MN, Bartolucci GB, Violante FS, Soleo L [2010]. Validity of new biomarkers of internal dose for use in the biological monitoring of occupational and environmental exposure to low concentrations of benzene and toluene. Int Arch Occup Environ Health *83*:341-356.

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