



S-Benzylmercapturic acid and S-phenylmercapturic acid in urine

8326

Metabolites of toluene and benzene

| | | | |
|------------------------------|--|-----------|-----------------|
| 1. S-Benzylmercapturic acid: | FORMULA: C ₁₂ H ₁₅ NO ₃ S | MW: 253.3 | CAS: 19542-77-9 |
| 2. S-Phenylmercapturic acid: | FORMULA: C ₁₁ H ₁₃ NO ₃ S | MW: 239.3 | CAS: 20640-68-0 |

METHOD: 8326, Issue 1 **EVALUATION:** Full **Issue 1:** 20 May 2014

Published limits and guidelines using these compounds as markers:

- 1. **Toluene:** OSHA and NIOSH: None
- 2. **Benzene:** OSHA and NIOSH: None

Other OELs: Because data on exposure limits and guidelines may change over time, NIOSH recommends referring to the following sources for updated limits and guidelines concerning the use of these compounds as markers for toluene and benzene [1- 4].

- PROPERTIES:** 1. Solid; density =1.246 g/cm³; mp 162-163 °C
2. Solid; density = 1.28 g/cm³; mp 155 °C

BIOLOGICAL

INDICATOR OF: Exposure to 1. toluene and
2. benzene

SYNONYMS (not all inclusive):

- 1. S-Benzylmercapturic Acid (BMA): S-benzyl-N-acetyl-L-cysteine; (2R)-2-Acetamido-3-(phenylmethylsulfanyl)propanoic acid; Alanine, N-acetyl-3-(benzylthio)-; S-phenylmethyl-N-acetyl-L-cysteine
- 2. S-Phenylmercapturic acid (PMA): S-phenyl-N-acetyl-L-cysteine; (2R)-2-Acetamido-3-(phenylsulfanyl)propanoic acid; (2R)-2-Acetylamino-3-(phenylthio)propionic acid

| SAMPLING | MEASUREMENT |
|--|--|
| <p>SPECIMEN: Urine</p> <p>VOLUME: At least 8 mL</p> <p>PRESERVATIVE: None added. Refrigerate or freeze upon collection.</p> <p>SHIPMENT: Ship cold or frozen with ice or dry ice. Freeze upon receipt at the laboratory.</p> <p>SAMPLE STABILITY: Stable in frozen urine for periods of a month or more and for several freeze/thaw cycles [5,6].</p> <p>CONTROLS: Urine specimens obtained from non-exposed or low level exposed individuals.</p> | <p>TECHNIQUE: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY (HPLC/MS/MS)</p> <p>ANALYTES: S-Benzylmercapturic acid and S-phenylmercapturic acid</p> <p>EXTRACTION: Solid-Phase Extraction (SPE) C18</p> <p>COLUMN: C18 [dimethyloctadecylsilane solid phase type, 3.5 µm particle size] (150 mm by 3 mm)</p> <p>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</p> <p>FLOW RATE: 0.3 mL/min (0.4 mL/min post run)</p> <p>GRADIENT: Time (min) vs. Mobile Phase Composition 0 to 10 0 to 40% B 10 to 18 40 to 100% B 18 to 20 100% B 20 to 21 100% B Flow increased to 0.4 mL/min 21 to 28 100% B (0.4 mL/min flow) 28 to 30 100 to 0% B (re-equilibration, 0.3 mL/min) 30 to 37 0% B (re-equilibration)</p> <p>INJECTION VOLUME: 8 µL</p> <p>IONIZATION SOURCE: Electrospray at 3500 Volts and negative scan mode, nebulizer gas at 35 psi and 10 L/min flow</p> <p>DETECTOR (MS/MS): Dwell time = 200 msec; Fragmentor at 80 Volts; Collision energy at 8 Volts; Collision gas: nitrogen at 0.06 L/min</p> <p>MULTIPLE REACTION MODE: Quantification mass transitions; BMA = m/z 252 → 123, PMA = 238 → 109, d₅-BMA = 257 → 128, d₅-PMA = 243 → 114</p> <p>TOTAL RUN TIME: Approximately 37 minute cycle time</p> <p>CALIBRATION: BMA and PMA solutions with internal standards</p> <p>QUALITY CONTROL: At least one level of spiked urine specimen prepared from a separately weighed stock solution</p> <p>RANGE: 0.5 to 50 ng/mL for BMA and PMA</p> <p>ESTIMATED LOD: Approximately 0.2 ng/mL for BMA and PMA; by lowest standard levels, BMA and PMA = 0.5 ng/mL</p> <p>PRECISION (\bar{S}_r): See Table 2</p> |
| ACCURACY* | |
| <p>RANGE STUDIED: See Table 2</p> <p>BIAS: Negligible</p> <p>OVERALL PRECISION (\hat{S}_r)*: See Table 2</p> <p>ACCURACY*: Overall recoveries obtained from spiked urine samples (n=48) were 103% and 106% for S-benzylmercapturic acid and S-phenylmercapturic acid, respectively. The precision as relative standard deviation was no greater than 5.0% at any concentration level (n=9, Table 2).</p> | |

* The definitions of precision and accuracy in this method are those utilized by the US Food and Drug Administration [7].

APPLICABILITY: BMA and PMA are metabolites of toluene and benzene, respectively. 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 [8]. 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.

INTERFERENCES: None found or identified.

OTHER METHODS: This method is based on the research of B'Hymer [9,10]. There are numerous literature methods for these two compounds, but standardized methods from governmental agencies or consensus standards organizations are not currently available, though there is also a recent method developed at CDC/National Center for Environmental Health for these analytes and others [11].

REAGENTS:

1. S-Benzylmercapturic acid (BMA, *N*-acetyl-S-benzyl-DL-cysteine, CAS no. 19542-77-9) reference standard 0.20 mg/mL stock solution in methanol. Store at 4 °C in amber vials (or in the dark).
2. S-Phenylmercapturic acid (PMA, phenyl-*N*-acetyl-DL-cysteine, CAS no. 20640-68-0) reference standard 0.20 mg/mL stock solution in methanol. Store at 4 °C in amber vials (or in the dark).
3. Deuterated S-benzyl-d₅-mercapturic acid reference standard (d₅-BMA), 0.15 mg/mL stock solution in methanol. Store at 4 °C in amber vials (or in the dark).
4. Deuterated S-phenyl-d₅-mercapturic acid reference standard (d₅-PMA), 0.15 mg/mL stock solution in methanol. Store at 4 °C in amber vials (or in the dark).
5. Internal standard spiking solution, deuterated stock solutions diluted to approximately 30 ng/mL in water
6. Acetone, HPLC grade or better*
7. Acetonitrile, HPLC grade or better*
8. Acetic Acid, glacial, ACS reagent grade or better*
9. Methanol, HPLC grade or better*
10. Water, doubly deionized, minimum resistivity of 18 MΩ-cm
11. Water, HPLC grade
12. Acetonitrile/water (50/50%, v/v) injector rinse solution
13. Chromatographic matrix adjustment solution, 49/50/1% (v/v/v) acetonitrile/water/acetic acid
14. Mobile phase A (5/95/0.1%, v/v/v) acetonitrile/water/acetic acid, filtered through 0.7 μm glass microfiber filters
15. Mobile phase B (75/25/0.1%, v/v/v) acetonitrile/water/acetic acid, filtered through 0.7 μm glass microfiber filters
16. Synthetic urine substitute, if desired
17. Nitrogen, UHP

* See SPECIAL PRECAUTIONS*

EQUIPMENT:

1. High-Performance Liquid Chromatograph (HPLC) equipped with a tandem mass spectrometric detector with data collection system, page 8326-1
2. HPLC column (C18 [dimethyloctadecylsilane solid phase type], 150 mm X 3 mm, 3.5 μm particle size)
3. Autosampler
4. Analytical balance, 0.1 mg readability
5. Rotary vacuum concentrator with cold trap and vacuum pump or analytical evaporator with nitrogen
6. Automatic pipet with disposable tips in the 1000 μL volume delivery range
7. Solid-phase extraction (SPE) cartridges, C18, 500 mg, 3 mL
8. SPE vacuum manifold apparatus
9. Culture tubes, disposable screw-top (16 X 150 mm) with PTFE-lined caps
10. Tubes, polypropylene, disposable screw-top, 15 mL. Polypropylene bottles and caps may also be used for sample collection.
11. Metal spatula
12. Volumetric flasks: 10, 50, 100 and 200 mL
13. Autosampler vials, amber, with caps and septa
14. Disposable glass pipets
15. Sintered glass filtration apparatus and 1-L side-armed Erlenmeyer flask
16. Microfiber filters, glass, 0.7 μm; diameter to fit the filtration apparatus
17. Wet or dry ice

SPECIAL PRECAUTIONS: Standard precautions should always be used when handling bodily fluids and/or extracts of bodily fluids [12]. Handle urine specimens and urine extracts using powder-free latex or nitrile gloves. Acetic acid, acetone, acetonitrile and methanol are flammable; handle with care and use in a chemical fume hood. Handle all chemicals using the required safety precautions. Reagents with manufacturer expiration dates should be observed.

SAMPLING:

1. Collect at least 8 mL of urine in an appropriate polypropylene tube or bottle and cap. Refrigerate at 4 °C or freeze after collection. Collect at least two urine specimens for each worker, one before the work shift and one after.
2. Ship the specimen stored in either wet or dry ice in an insulated container. Freeze specimens upon arrival at the laboratory and store frozen. A reminder: commercial shippers have special labeling requirements for packages containing dry ice.

SAMPLE PREPARATION:

NOTE: BMA and PMA are somewhat light sensitive (see Evaluation of Method section). Perform the sample preparation steps in a low light environment. Extreme measures are not required.

3. Thaw the urine specimen to room temperature.
4. Mix urine specimen thoroughly to ensure homogeneity.
5. Transfer 4.0 mL of urine into a 16 X 150 mm (or larger) screw-capped culture tube.
6. Add 0.5 mL of deionized water to aid in the dissolution of solids.
7. Add 0.5 mL of the 30 ng/mL deuterated BMA/PMA internal standard solution.

8. Solid-phase extraction

NOTE: All SPE steps are performed using the vacuum manifold apparatus. The flow rate should not exceed 1 mL/min. The SPE cartridges should not be allowed to go to dryness until the end of Step 8d. Refer to manufacturers' recommendations for use of specific SPE cartridges.

- a. Pre-wash the C18 SPE cartridge with 2 mL of acetone.
 - b. Equilibrate the SPE cartridge with 2 mL of HPLC grade water.
 - c. Load the 5-mL urine mixture and draw the sample through the cartridge.
 - d. Wash the cartridge with 1 mL of HPLC grade water. Discard any liquid collected up to this point.
 - e. Apply (or increase) the vacuum to pull most of the water from the cartridge.
 - f. Elute the analytes with 3 mL of acetone three times, collecting all of the acetone washes into a 15-mL plastic screw-capped tube.
9. Evaporate the 9 mL of acetone from the extracts to dryness by means of a vacuum rotary concentrator or by using a nitrogen sweep.
 10. Cap the plastic tubes containing the dry extract and store in a refrigerator/freezer until ready for chromatographic analysis.
 11. Prior to chromatographic analysis, dissolve the extract in 1 mL of HPLC mobile phase A and transfer the sample into an amber HPLC autosampler vial.

CALIBRATION AND QUALITY CONTROL:

12. Prepare a BMA/PMA standard mixture by combining 2.0 mL of each stock solution and diluting to 100 mL with deionized water to make an approximately 4 µg/mL solution concentration for each.
13. BMA/PMA standard solutions: The 4 µg/mL BMA/PMA solution is diluted in water to make 4, 8, 16, 40, 80, 320 and 400 ng/mL BMA/PMA solutions.
14. Accurately transfer 0.500 mL of each BMA/PMA solution from step 13 into a separate HPLC autosampler vial. [This delivers 2, 4, 8, 20, 40, 160 and 200 ng of each analyte to each autosampler vial.]
15. Add 0.5 mL of the deuterated internal standard spiking solution [30 ng/mL] to each vial. [This delivers 15 ng of the internal standards to each autosampler vial.]

16. Add 0.1 mL of the chromatographic matrix adjustment solution to each standard vial.
NOTE: These standard samples are *equivalent* to 0.5 to 50 ng/mL BMA/PMA urine samples [based on the original 4.0 mL urine volume extracted and placed in each autosampler vial.]
17. Prepare one blank urine sample without analyte spikes; alternatively, prepare one blank using a urine substitute by following steps 14-16 using 0.5 mL of the unspiked urine or substitute in step 14.
18. Prepare at least one quality control (QC) standard of urine or urine substitute fortified with PMA/BMA using separately prepared stock solutions. A 10 ng/mL equivalent spike level is suggested and more than one level can be used if desired.

MEASUREMENT:

19. Set the high-performance liquid chromatograph according to the manufacturer's recommendations and to the conditions listed on page 8326-1. A needle rinse with 50/50% (v/v) acetonitrile/water is required to eliminate sample carry-over by the autosampler.
20. Set the mass spectrometric detector to multiple reaction mode (MRM) according to the manufacturer's recommendations and the conditions listed on page 8326-1. Example conditions are summarized below:

Table 1. MS/MS Conditions using negative electrospray ionization

| 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 |

21. Inject 8 µL of each sample extract, standard, QC standard, and blank. Sample chromatograms for each compound are illustrated in Figure 1.
22. Measure the peak areas of the two analytes (BMA and PMA) and those for the deuterated internal standards (d₅-BMA and d₅-PMA) in the chromatograms. Divide the peak area of the analytes by the peak area from the matching deuterated internal standard.
23. Prepare calibration curves of the peak Area Std./Area Internal Std. (ratio calculated in step 22) versus the *urine equivalent* concentration of the standards for the two analytes.

CALCULATIONS:

24. Determine the concentration of the two analytes in the extracts from the original urine (4.0 mL specimen) from the curves obtained in step 23. The results can be expressed as ng/mL of each analyte in urine.

EVALUATION OF METHOD:

This method was evaluated and described in detail by B'Hymer [9,10]. A general summary of this published information is given below:

Accuracy and Precision. Three recovery studies using multiple columns over several days demonstrated the accuracy and precision of this test method. The first recovery study was performed using fortified urine samples over three separate experimental batch runs, and these data are presented in Table 2. Average recoveries were between 102 and 106% for the two analytes over the four spiked

concentration levels investigated. For each batch run, the experimental trial consisted of three samples at four different concentration levels. The recovery and precision for each level ($n = 9$ samples) are displayed in Table 2. The relative standard deviations (RSD) observed ranged from 2.0 to 5.0%. These accuracy and precision numbers fall well within parameters established for bioanalytical methods [7].

The second recovery study was performed using a urine substitute, fortified over three separate experimental batch runs, and these data are presented in Table 3. Average recoveries were between 99 and 109% for the two analytes over the four spiked concentration levels investigated. For each batch run, the experimental trial consisted of three samples at four different concentration levels. The recovery for each level ($n = 9$ samples) is displayed in Table 3. As seen in the RSD column of Table 3, precision was much worse for the urine substitute recovery study. This is due to a higher level of ion suppression in the electrospray source. This was especially pronounced at the lower spiked concentration levels (1 and 2 ng/mL). The highest result for the RSD of the 9 samples fortified at 1 ng/L BMA was 29%. While the accuracy numbers meet standard criteria, the precision values at the lower concentrations do not [7]. The use of a urine substitute for QC samples is not recommended for this method.

The third recovery study used urine specimens from twelve non-exposed volunteers; six were smokers and six were non-smokers. Most participants' specimens had base levels for both BMA and PMA as are shown in Table 4. Again, reasonable accuracy and precision were demonstrated for a 6 ng/mL equivalent level spike; individual recoveries ranged from 95 to 109% for BMA and 101 to 123% for PMA (Table 4). No interferences were detected in the unspiked urine from the 12 volunteer specimens. This study illustrates two important points: 1. Levels of both metabolites can be found in the urine of non-exposed individuals, which is why aqueous standards are used in this method, and 2. It is important to ascertain smoking status and other non-occupational exposures that may cause elevated levels of these metabolites.

Linearity. All calibration curves used during the development of this method were linear and had correlation coefficients of 0.99 and greater. The concentration range was equivalent to 0.5 to 50 ng/mL BMA and PMA for the extraction of 4.0 mL urine. Calibration curves were run at the beginning and end of all sample batch runs; calibration curve slope drift was found to be minimal.

Specificity. The optimized chromatographic conditions developed for this method, along with the tandem mass spectrometric detector, proved to be specific and showed no major interferences. The mass transition ions of BMA and PMA chosen in this method had the greatest response and were the predominant daughter ions.

Robustness. Two C18 HPLC columns (Zorbax Rx-C18, Agilent Technologies, Santa Clara, CA, USA) from the same manufacturer but from different manufacturing lots were used during the recovery studies. Accuracy and precision were not affected; therefore, the method was found to be reproducible with any normal functioning C18 HPLC column. Recovery results from individual volunteer urine specimens spiked with the analytes indicate that the method is accurate and not significantly affected by individual urine specimen matrix differences during analyte extraction. The urine substitute was found to cause problems with precision, especially at lower levels; this was attributed to an increase in ion suppression over human urine within the electrospray source.

Stability. Sample stability was evaluated, whereby a six-day stability study was conducted on the final chromatographic sample solution. BMA and PMA were stable at 8 °C (the autosampler temperature) and at room temperature in the absence of light. A light stability experiment was carried out by storing this solution in a clear glass vial at room temperature on the window sill, thus creating a worst-case scenario. After 1 day of storage in light under these conditions, 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 fallen 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 recommended to ensure sample stability during extended chromatographic batch runs.

Range. This method should be considered accurate for the estimation of BMA and PMA in human urine within the 0.5 to 50 ng/mL standard curve range. Field samples at higher levels can be diluted to a concentration within that range for analysis.

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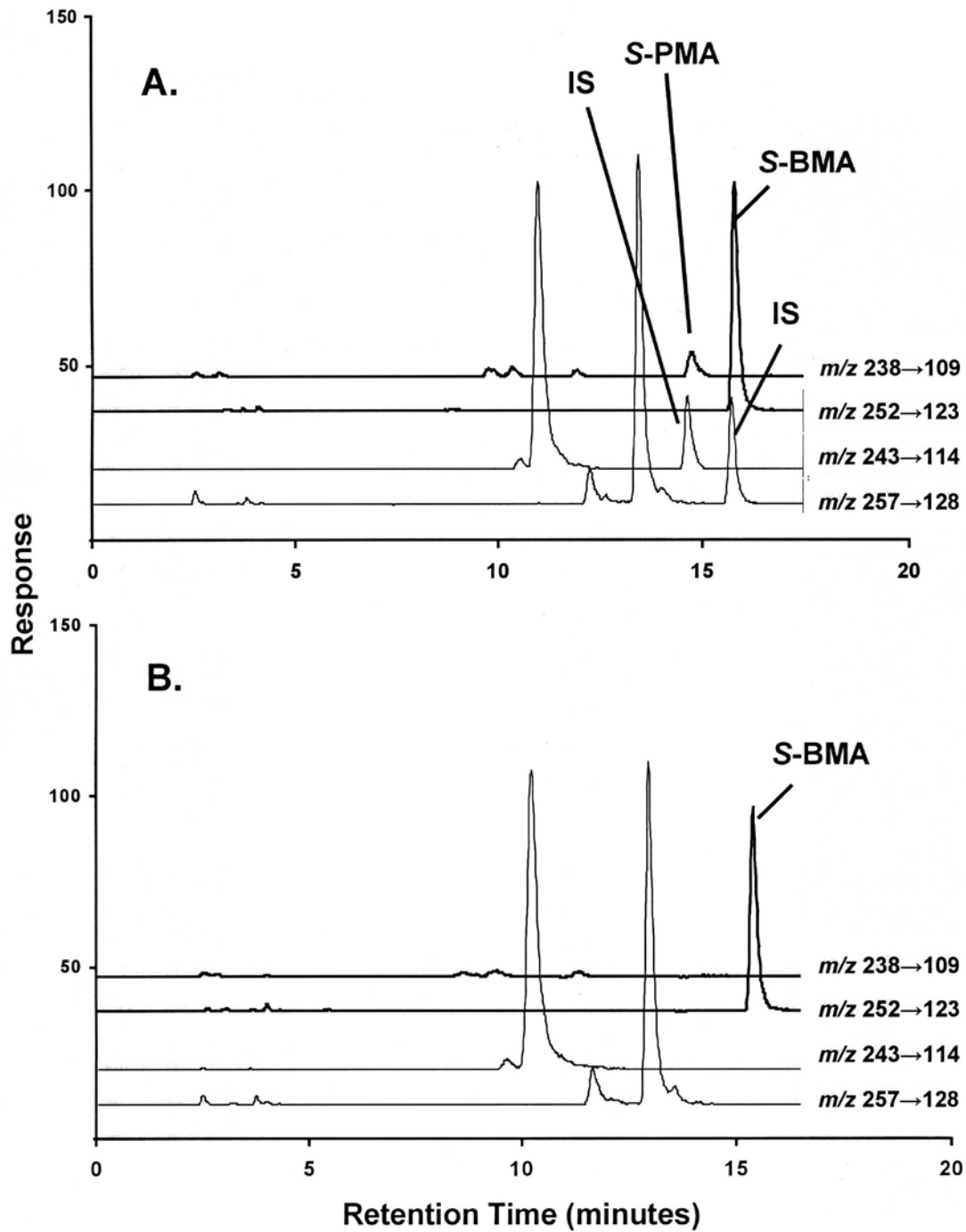


Figure 1. Chromatograms obtained using the described procedure: (A) fortified urine containing 1 ng/mL S-PMA, 7.2 ng/mL S-BMA, and 3.8 ng/mL of each of the deuterated internal standards (IS) and (B) non-fortified urine from above containing only the background level of 6.2 ng/mL S-BMA

Table 2. Multiple level recovery experiment of BMA and PMA from urine

| Analyte | Analyte added (ng/mL) | Mean concentration (ng/mL, n = 9) | Mean background corrected concentration (ng/mL, n = 9) | Average recovery (%) | Standard deviation (ng/mL) | %RSD ¹ |
|------------------|-----------------------|-----------------------------------|--|----------------------|----------------------------|-------------------|
| BMA ² | 1 | 7.43 | 7.18 | 103 | 0.37 | 5.0 |
| BMA ² | 2 | 8.44 | 8.18 | 103 | 0.19 | 2.3 |
| BMA ² | 8 | 14.6 | 14.2 | 103 | 0.32 | 2.2 |
| BMA ² | 30 | 38.2 | 36.2 | 106 | 0.94 | 2.4 |
| PMA | 1 | 1.02 | 1.00 | 102 | 0.05 | 4.9 |
| PMA | 2 | 2.10 | 2.00 | 105 | 0.09 | 4.3 |
| PMA | 8 | 8.23 | 8.00 | 103 | 0.27 | 3.3 |
| PMA | 30 | 31.9 | 30.0 | 106 | 0.65 | 2.0 |

Notes: 1. %RSD = percent relative standard deviation

2. The non-fortified reference urine had a background level of 6.2 ng/mL BMA and no detectable level of PMA.

Three different spiked samples were prepared at each level and analyzed during three separate experimental trial runs (a total of nine samples at each spike level were analyzed). The same C18 column was used for experimental batch trials 1 and 2; a second C18 column was used on trial run 3.

Table 3. Multiple level recovery experiment of BMA and PMA from a urine substitute

| Analyte | Analyte added (ng/mL) | Mean concentration (ng/mL, n = 9) | Average recovery (%) | Standard deviation (ng/mL) | %RSD ¹ |
|---------|-----------------------|-----------------------------------|----------------------|----------------------------|-------------------|
| BMA | 1 | 1.07 | 107 | 0.31 | 29 |
| BMA | 2 | 2.13 | 106 | 0.34 | 16 |
| BMA | 8 | 8.31 | 104 | 0.53 | 6.4 |
| BMA | 30 | 30.7 | 102 | 0.76 | 2.5 |
| PMA | 1 | 1.09 | 109 | 0.27 | 25 |
| PMA | 2 | 1.98 | 99 | 0.16 | 7.9 |
| PMA | 8 | 7.96 | 100 | 0.48 | 6.0 |
| PMA | 30 | 31.1 | 104 | 0.91 | 2.9 |

Notes: 1. %RSD = percent relative standard deviation.

Three different spiked samples were prepared at each level and analyzed during three separate experimental trial runs (a total of nine samples at each spike level were analyzed).

Table 4. Individual smoker and non-smoker recovery experiment of BMA and PMA

| Individual Sample | Background Level | | Fortified Urine (background + 6 ng/mL) | | Fortified Urine Recovery | |
|-------------------|------------------|-------------|---|-------------|--------------------------|-----------------------|
| | BMA (ng/mL) | PMA (ng/mL) | BMA (ng/mL) | PMA (ng/mL) | BMA [ng/mL (percent)] | PMA [ng/mL (percent)] |
| Smoker 1 | 2.7 | 0.2 | 8.7 | 6.2 | 8.6 (99%) | 6.5 (104%) |
| Smoker 2 | 28.3 | 0.3 | 34.3 | 6.3 | 34.7 (101%) | 6.9 (108%) |
| Smoker 3 | 15.9 | 0.9 | 21.9 | 6.9 | 21.3 (97%) | 7.9 (114%) |
| Smoker 4 | 5.7 | 0.3 | 11.7 | 6.3 | 11.6 (99%) | 7.7 (121%) |
| Smoker 5 | 1.3 | nd | 7.3 | 6.0 | 6.9 (95%) | 6.4 (106%) |
| Smoker 6 | 9.2 | 0.7 | 15.2 | 6.7 | 15.5 (102%) | 7.0 (104%) |
| Mean | 10.5 | 0.4 | 16.5 | 6.4 | 16.4 (99%) | 7.1 (110%) |
| Non-smoker 1 | 0.3 | nd | 6.3 | 6.0 | 6.2 (98%) | 6.2 (103%) |
| Non-smoker 2 | 7.1 | nd | 13.1 | 6.0 | 14.3 (109%) | 7.4 (123%) |
| Non-smoker 3 | 6.8 | nd | 12.8 | 6.0 | 13.1 (102%) | 6.8 (112%) |
| Non-smoker 4 | 23.3 | nd | 29.3 | 6.0 | 28.6 (97%) | 6.3 (105%) |
| Non-smoker 5 | 4.7 | nd | 10.7 | 6.0 | 11.0 (103%) | 6.3 (105%) |
| Non-smoker 6 | 7.2 | nd | 13.2 | 6.0 | 13.5 (102%) | 6.1 (101%) |
| Mean | 8.2 | - | 14.2 | 6.0 | 14.5 (102%) | 6.5 (108%) |

Notes: The instrumental limit of detection (LOD) was estimated to be approximately 0.2 ng/mL for both analytes. The lowest standard value was 0.5 ng/mL for both analytes. [Values near the LOD were reported to one significant figure.]
 nd = none detected (< 0.2 ng/mL)