BACKUP data for 3-BROMOPROPIONIC ACID in URINE

Metabolite of 1-Bromopropane

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Substance: 3-Bromopropionic acid, 3-BPA

Exposure Limits: Not applicable/Not available

Chemical Used for Evaluation: 3-BPA was obtained from Sigma-Aldrich Chemical Corporation (St. Louis, Missouri, USA).

GENERAL

Synopsis

The compound, 3-bromopropionic acid (3-BPA), is a metabolite of 1-bromopropane and is a proposed biomarker of exposure. 1-Bromopropane has several industrial applications including its use as a component in adhesives. It also has many other industrial applications including cleaning metal, optical instruments and electronics. The described procedure was developed for the detection and quantification of free 3-BPA in urine. Urine from an exposed population is collected, shipped cold and stored frozen at -80 ºC. The samples are thawed for analysis and acidified. Liquid-liquid extraction (LLE) using ethyl acetate collects the 3-BPA. The extracts are reduced in volume and then silylated by the use of N-methyl-N-[tert-butyldimethylsilyl]trifluoroacetamide to produce the corresponding tert-butyldimethylsilane derivative. Measurement is by means of a gas chromatograph (GC) equipped with a mass selective detector (MSD) using a dimethylpolysiloxane (HP-1, or equivalent) capillary column. 3-Chloropropionic acid (3-CPA) is used as the internal standard.

Method Evaluation

The method was evaluated in the general areas of a typical method validation. [1-3] Furthermore, this method has been described by B'Hymer [4] and published by B'Hymer and Cheever. [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 recovery studies 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 spiked urine samples were prepared by adding 0.5 mL of the appropriate aqueous spike level solution to the 2.0 mL aliquot of urine. Non-spiked samples, including the actual field samples, are diluted with 0.5 mL of water. All samples are spiked with a 0.5 mL aliquot of 20 µg/mL 3-chloropropionic acid in water, which is the internal standard. Solids found in urine samples were found not to cause a problem with the liquid-liquid extraction; therefore, filtering the urine was not included in this test method's sample preparation.

Analytical Aspects

Instrumental Parameters

The chromatographic analysis was carried out using an Agilent Technologies model 6890 gas chromatograph (Palo Alto, California, USA) equipped with a model 5973 mass selective detector and an autosampler. The detector output was connected to a Chemstation (Agilent Technologies) where all raw data were evaluated and integrated. The column was a capillary HP-1 (Agilent Technologies) with a length of 50 m, internal diameter of 0.20 mm and film thickness of 0.33 µm. The instrumental conditions for analysis were as follows: helium carrier flow was 0.8 mL/min constant, injector port temperature was 200 °C, and the detector source temperature was 230 °C with the quadrupole set at 150 °C. The column program was as follows: the initial temperature was 60 °C, then increased to 180 °C

at a rate of 4 °C/min and finally increased to 255 °C at a rate of 15 °C/min. A post run of 270 °C for five minutes was included with each run. The mass selective detector was operated in electron ionization mode with an electron energy of 70 eV and selected ions were monitored at ion m/z 211 (3-BPA derivative) and ion m/z 165 (3-CPA derivative) for quantification. Also, the mass selective detector was used in the scanning mode for verification of the identity of peaks during the initial development phase of this analysis procedure. Example full-scan mass spectra of both derivatized analytes as well as a typical selected-ion chromatogram can be found in method. The injection size of the final solution was 0.5 µL using splitless mode injection.

Extraction/Sample Preparation

Non-spiked urine samples or 3-BPA-spiked blank urine samples were treated identically. A 2.0-mL portion of the urine was placed in a glass screw-capped tube and acidified with 40 µL of concentrated (12M) hydrochloric acid. A 0.5 mL aliquot of a 20 µg/mL of 3-CPA internal standard solution was added. A 0.5 mL portion of deionized water for test samples or standard 3-BPA spiking solution was added. The urine sample was extracted four times with 4 mL of ethyl acetate using a vortex mixer for one minute for each extraction. The ethyl acetate layers were combined and dried with anhydrous magnesium sulfate and filtered through silanized glass wool. It is important that the glass wool be packed tightly enough to prevent magnesium sulfate particles from passing through. The combined solution was reduced in volume to 1 mL by evaporation using a nitrogen sweep at room temperature. The urine extracts were transferred to autosampler vials, treated with 50 µL of the silylation reagent, N-methyl-N-[tert-butyldimethylsilyl]trifluoroacetamide (MTBSTFA) with 1% tert-butyldimethylchlorosilane (TBDMCS), for ninety minutes at 70 oC. These solutions were then analyzed by the GC/MS.

The chemical reagents used were those commonly found in a laboratory. Ethyl acetate was HPLC grade. All other reagents were ACS reagent grade. The internal standard compound, 3-chloropropionic acid (3-CPA), was available commercially (Sigma-Aldrich). The derivatizing reagent, MTBSTFA with 1% TBDMCS, was also commercially available (Sigma-Aldrich).

Method developmental considerations

The ethyl acetate extraction efficiency has been determined to be greater than 80% for 3-BPA. This was estimated by measuring the response of extracted solutions against known concentration solutions of 3-BPA derivative. Since a low detection limit was desired for this method, high extraction efficiency was considered important. Silylation to the tert-butyldimethylsilane (TBDMS) derivative proved to be effective, simple and was a derivatization procedure which was not complicated. 3-Chloropropionic acid was chosen as the internal standard for a number of reasons. Generally, a worker would not be expected to be exposed to 1-chloropropane and the acid metabolite should not be present in urine. An internal standard compensated for changes in solvent volume, however, the use of 3-CPA as a procedural internal standard reduced analysis and extraction variation to acceptable levels. 3-CPA is chemically similar to 3-BPA and has similar solubility and extraction properties. Other internal standards were evaluated during the development stage of this method. 4-Bromobutyric acid was found to not extract at a high yield using ethyl acetate and was dropped from consideration The deuterated analog of 3-BPA was tried with greater success. The recovery and early calibration curves generated using deuterated 3-Bromopropionic acid as an internal standard were as good as those using 3-CPA. However, due to the high cost of using a deuterated analog, 3-CPA was chosen as the internal standard for the final test method. The chromatographic conditions described will resolve the deuterated analog for the 3-BPA derivative peak should an alternative internal standard be required or desired in the future.

Results

Accuracy and Precision

Two recovery studies using multiple columns over several days demonstrated the accuracy and precision of this test method. The recovery studies were performed using spiked urine samples containing known levels of 3-BPA. The first recovery study was performed over three separate

experimental batch runs, and these data are presented in Table 1A. Average recovery was between 95 and 103% for the three 3-BPA spiked level urine samples investigated. For each batch run, the experimental trial consisted of three samples at three different concentration levels. The recovery for each level (n=9 samples) is displayed in Table 1A. This initial recovery study was used to establish the general accuracy and precision of the method. The second recovery study used urine samples from 20 non-exposed volunteers and demonstrated that the procedure was accurate and precise (Table 1B). No interferences were detected in the non-spiked urine from the 20 volunteer samples. Furthermore, this second study verified that any urine sample matrix difference among individuals did not significantly affect the accuracy or precision of the method. Both recovery studies generated a total of 47 spiked urine samples at 2, 10, 20 and 50 µg/mL 3-BPA levels; thus, four different levels of 3-BPA for spiked samples were studied. Precision expressed as percent relative standard deviation (%RSD) was 5.7% on the 2 µg/mL recovery samples (n=9). This was the highest level of %RSD at any of the four concentration levels studied. Overall recovery was 95% and overall RSD was 3.1% for all 47 spiked urine samples. Bias can be assumed to be very small or insignificant. These accuracy and precision values are well within limits recommended for bioanalytical methods. [3]

Linearity

All calibration curves used during the development of this method were linear and had correlation coefficients of 0.98 and greater. The concentration range was 0.1 to 200 µg/mL 3-BPA in urine with 2.0 mL urine sample size. Calibration curves were run at the beginning and end of all sample batch runs; calibration curve slope drift was found to be acceptable.

Specificity

The optimized chromatographic conditions developed for this procedure proved to be specific and have no major interferences. The mass selective detector was useful in adding additional specificity to the method. The ion m/z 211 was chosen for monitoring the calibration curve used in the calculations because of its greater abundance, and it was a characteristic fragment for the TBDMS derivative of 3-BPA. This is the molecular ion less the tert-butyl group, m/z 57 (See Figure 1). Ion m/z 165 was used to monitor the TBDMS derivative of 3-CPA, the internal standard, for the same reasons. Further discussion regarding the use/non-use of qualifier ions for this method is published elsewhere. [5]

Robustness

Multiple HP-1 columns of different manufacturing lots 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 HP-1 capillary column. Recovery results from individual urine samples spiked with 3-BPA indicates that the method was accurate and not significantly affected by individual urine sample matrix differences during analyte extraction.

Stability

Sample stability was not exhaustively evaluated. Aqueous stock standard solutions of 3-BPA stored for two weeks gave full recovery assay values when compared to a freshly prepared 3-BPA standard. Samples of derivatized 3-BPA appeared to be stable during a one week time frame.

Range

This method should be considered accurate for the estimation of 3-BPA in human urine within a 2.0 to 100 µg/mL range. The recovery studies reported here (Table 1) tested down to the lower level of 2.0 µg/mL, while the User Check studies tested up to the highest level of 100 µg/mL.

Limit of detection

The limit of detection (LOD) was calculated in the traditional way, three times the noise level of a typical blank (non-spiked) urine divided by the slope of the calibration curve. [1] The noise level was determined for several batch runs by determining the average noise level for 100 baseline points in the retention time window for the 3-BPA derivative from blank urine preparations. The slope from the calibration curve using 3-BPA peak height of the standards was used for this calculation. Different columns at various conditions and ages were evaluated to calculate the LOD. The limit of detection

was found to be approximately 0.01 µg/mL equivalent levels of 3-BPA in urine. Columns near the end of their useful lifespan had 3-BPA derivative peaks with lower theoretical plate counts and more peak tailing than newer columns. Variation in the signal-to-noise ratio of the mass selective detector also played a role in the calculated LOD. This method calls for a lowest standard concentration of 0.1 µg/mL, which can be considered an "operational" LOD and a basic criterion for using the method. If a column or chromatographic system cannot detect the lowest level standard, corrective action would be required.

Ruggedness

Laboratory-to-laboratory reproducibility was not evaluated for this method. This method was originally developed for support of 1-bromopropane field studies within the Biomonitoring and Health Assessment Branch (BHAB); field study samples analyzed to date of this report have revealed no problems with this method. User Check recoveries for blind-spike samples ranged from 95-101% over four concentration levels with an overall average of 98.3% recovery. The total precision as well as the precision at each level was less than 5% relative standard deviation. These data can be found appended to the end of the report.

Table 1. Recovery studies of 3-bromopropionic acid: (A) multilevel recovery study of 3-BPA from spiked urine samples[a], (B) recovery of 20 µg/mL 3-BPA spikes from urine samples of 20 non-exposed volunteers[b]

(A) Spike level (µg/mL)	Mean 3-BPA recovered (n = 9, µg/mL)	Average % Recovery	SD (µg/mL)	% RSD
2	1.91	96	0.11	5.7
10	9.32	93	0.13	1.4
50	48.9	98	0.36	0.7
(B) Volunteer urine Spike level (µg/mL)	Mean 3-BPA recovered (n=20, µg/mL)			
20	19.0	95	0.48	2.5

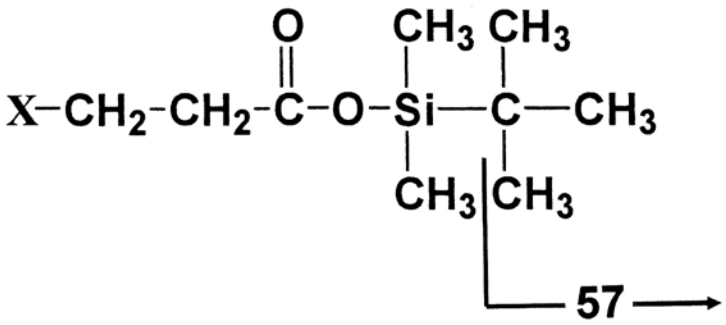
[a] Three different spiked urine samples were prepared at each level and chromatographed on three separate experimental trial runs (a total of nine samples at each spike level were analyzed).

[b] All non-spiked samples showed no 3-BPA derivative peak in the chromatograms.

SD = standard deviation. % RSD = percent relative standard deviation.

Note: Overall recovery of all samples was 95% and overall RSD was 3.1% (n = 47).

Figure 1



X = ^{35}Cl, m/z = 165 or for ^{37}Cl, m/z = 167.

X = ^{79}Br, m/z = 209 or for ^{81}Br, m/z = 211.

The major ions monitored for the tert-butyldimethylsilane derivatives were the molecular ions less the tert-butyl group (m/z = 57). Ion of m/z = 165 for the internal standard derivative and ion of m/z = 211 for the 3-bromopropionic acid derivatives were used for quantitation.

References

[1] J.M. Green, "A practical guide to analytical method validation," Anal. Chem. 68 (1996) A305-A309.
[2] International Conference on Harmonisation, "Draft guidelines on validation of analytical procedures: definitions and terminology," The Federal Register 60 (1995) 11260.
[3] U.S. Food and Drug Administration. "Guidance for Industry: Bioanalytical Method Validation." US FDA, May 2001.
[4] C. B'Hymer, "Determination of 3-bromopropionic acid in Urine," NIOSH, Division of Applied Research and Technology, Biomonitoring and Health Assessment Branch SOP no. E3-64, June 2003.
[5] C. B'Hymer, K.L. Cheever, "Development of a gas chromatographic test for the quantification of the biomarker 3-bromopropionic acid in human urine," J. Chromatogr. B, 802 (2004) 361-366.

APPENDIX

Review of User Check for NMAM Method 8324 (3-Bromopropionic acid in urine)

User check samples were prepared by a BHAB researcher (Dr. Clayton B'Hymer) to be analyzed by ALS Environmental using draft NMAM Method 8324. A total of 25 urine samples were prepared. The urine was obtained from personnel in the Taft building at NIOSH and then combined and mixed in the BHAB labs into a single pool of urine from which all samples were prepared. The final volume of each sample was 10 mL. Five samples were left blank. Five samples were prepared containing the analyte at each of the following levels: 2.51 µg/mL, 5.02 µg/mL, 60.22 µg/mL, and 100.4 µg/mL. The samples were shipped frozen to ALS Environmental on January 30, 2013 and arrived there the next day. The samples were analyzed on February 20, 2013. No significant deviations from the analytical procedure in NMAM Method 8324 were noted.

For this analysis, the Reporting Limit (RL, which is equivalent to the limit of quantitation, LOQ) was determined by ALS to be 0.10 µg/mL. As mentioned above, the spike levels ranged from 2.51 to 100.4 µg/mL which is 25 to 1000 times the RL and fall within the linear range of the calibration curve.

The complete data set from the User check samples is given following this review. The analyte was not detected in any of the blank urines, which is to be expected, so no blank correction is required in the method nor was it required in this set of samples. A summary table for each level is shown below.

Spiked concentration (µg/ mL)	Compound	Average Recovery (%)	RSD (%)
2.51	3-BPA	95.5	4.9
5.02	3-BPA	101.3	2.3
60.22	3-BPA	100.9	3.6
100.4	3-BPA	95.4	4.1
Overall	3-BPA	98.3	4.8

The recovery accuracy at every level is within +/- 5% of the true value which is well within the +/- 15% accuracy required for bioanalytical methods by the US Food and Drug Administration [3]. The relative standard deviation (RSD, which is a measure of precision) for all levels ranged from 2 to 5 per cent, which is also well within acceptable limits. The contract lab reported no difficulties understanding the draft method nor in setting it up or analyzing the samples. This method is lengthy and labor intensive. The User Check laboratory followed the procedure, but also did additional experiments and has made recommendations that will be taken into consideration for the final version of the method. The method has been shown to have adequate precision and accuracy. It is recommended that the method, NMAM Method 8324 (3-Bromopropionic acid in urine) be approved and accepted for inclusion in the NIOSH Manual of Analytical Methods.

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April 30, 2013

Method 8324 User Check complete data set

Sample Number	3-BPA found	Target	Recovery (%)	Level average	Level StDev	Level RSD
8	0.05	Blank				
13	0.05	Blank				
17	0.04	Blank				
18	0.05	Blank				
24	0.22	Blank				
1	2.34	2.51	93.2			
3	2.52	2.51	100.4			
5	2.26	2.51	90.0			
20	2.31	2.51	92.0			
25	2.55	2.51	101.6	95.5	4.7	4.9
6	5.22	5.02	104.0			
9	4.99	5.02	99.4			
19	5.12	5.02	102.0			
21	5.18	5.02	103.2			
22	4.91	5.02	97.8	101.3	2.3	2.3
2	63.76	60.22	105.9			
11	61.83	60.22	102.7			
12	59.16	60.22	98.2			
14	57.52	60.22	95.5			
23	61.65	60.22	102.4	100.9	3.6	3.6
4	101.46	100.4	101.1			
7	97.99	100.4	97.6			
10	89.56	100.4	89.2			
15	95.00	100.4	94.6			
16	95.07	100.4	94.7	95.4	3.9	4.1
		Ave Rec	98.3			
		Ave StDev	4.7			
		Ave RSD	4.8			

All concentrations are in μg/mL (mg/L).