# Development of a Gas Chromatographic Test for the Quantitation of the Biomarker 2-Butoxyacetic Acid in Urine Samples*

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## Abstract

**An accurate and precise method is developed and evaluated for the detection and quantitation of 2-butoxyacetic acid (2-BAA), a metabolite and biomarker for human exposure to 2-butoxyethanol. The solvent 2-butoxyethanol (2-BE) is extensively used in various industrial and domestic applications, and it is a health concern owing to its toxicity. Sample preparation consists of liquid–liquid extraction (LLE) of urine, then esterification of 2-BAA to produce the ethyl ester analog. The gas chromatographic conditions utilize a dimethyl polysiloxane phase (HP-1) capillary column and a mass spectrometer (MS) for detection of the analyte. Validation of this method includes a recovery study using fortified urine samples, which demonstrated good accuracy and precision; recovery varied between 100% and 102% of theory, with relative standard deviations of replicate samples at 2.8% and less. The detection limit of this method ranges from 0.005 to 0.015 µg/mL equivalent level of 2-BAA in urine.**

## Introduction

The compound 2-butoxyethanol (2-BE) has many industrial uses and is a component in many commonly used formulations. Its main use is in paints (1), surface coatings (2), and inks, but it is also used in numerous household cleaning products and aerosols (3,4). Its primary urinary metabolite in both humans and animals is 2-butoxyacetic acid (2-BAA); the structure for 2-BAA is shown in Figure 1. This makes 2-BAA a useful biomarker of exposure, which is the primary interest of this laboratory. 2-BE is a glycol ether; this class of compounds has been known for their toxicological properties for many decades (5), and their toxicity has been frequently reported in the literature (6,7). In animal studies, toxicological findings have included hemolysis, skin irritation, and decreased body weight (8). In the rat, 2-BE caused severe acute hemolytic anemia, as well as hemoglobinuria and histopathologic changes in the liver and kidney (9). Immune response alterations have been reported in mice from 2-BE topical exposure application (10).

2-BAA is best suited for the use as a urinary biomarker for short-term exposure to 2-BE; an outline of the metabolic pathway is shown in Figure 1. In general, 2-alkylethanol compounds are rapidly metabolized via alcohol dehydrogenase to the corresponding alkoxyacetic acids which can be further metabolized by various mechanisms such as acyltransferase and dealkylase carboligase (11). 2-BE may also be metabolized by glucuronyl transferase, dealkylase, or sulfotranferase, but the primary route leads to 2-BAA as was described previously. The analysis work reported in this manuscript is focused on the development and evaluation of a test to be used in the detection and quantitation of 2-BAA in human urine.

Alkoxyacetic acid analysis, including 2-BAA analysis, repre-

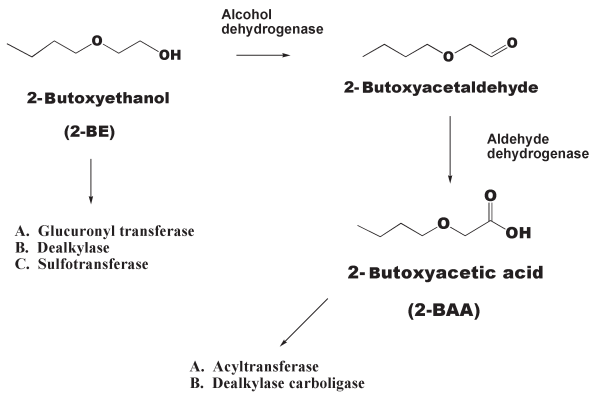


**Figure 1.** The metabolic pathways for 2-butoxyethanol (2-BE) leading to the formation of 2-butoxyacetic acid (2-BAA), a biomarker of exposure. 2-BE can also be metabolized by glucuronyl transferase, dealkylase and sulfotransferase. 2-BAA can be further metabolized by acyltransferase or dealkylase carboligase.

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sents an analytical challenge for chemists, and numerous procedures have been reported in the literature over the past few decades. Although the analysis of many urinary metabolites have used high-performance liquid chromatography (HPLC), many alkylacetic acids have been analyzed by gas chromatography (GC) (12–22) or by a radiolabeling approach for tracing metabolites. As with the monitoring of any biomarker of exposure, the lowest practical detection limit is desired for any quantitative test procedure as well as a high level of accuracy for results. Also, the absence of interferences with the test procedure is desired. Analyte extraction, concentration, and cleanup are important facets for any urinary biomarker analysis procedure. Most of the reported analysis procedures for alkoxyacetic acid metabolites formed from glycol ethers have utilized liquid–liquid extraction (LLE) followed by derivatization of the acid metabolite for analysis by GC. LLE has been noted in the literature for its inherent simplicity and ease of use over solid-phase extraction (SPE) (23), but LLE is often more labor intensive. Brown et al. (12) reported the use of SPE for extraction of 2-BAA from urine; however, SPE has demonstrated generally poor recovery results for alkoxyacetic acids including 2-BAA (24). Therefore, LLE was used for the current evaluation and test procedure development. Other GC alkoxyacetic acid urine analysis methods reported in the literature have followed extraction with derivatization using diazomethane (14), pentaflurobenzyl bromide (15–17), trimethysilyldiazomethane (18), *tert*-butyldimethylsilane (24), or esterification (20,24,25). Simple acid catalyzed esterification is generally less complicated than the other methods mentioned, and it uses less expensive reagents. Diazomethane is hazardous for the laboratory chemist, and pentafluorobenzyl bromide derivatization can generate irritating bromide gas. Although Shih et al. (19) first reported using direct GC analysis of 2-BAA without the derivatization step, this technique has been discounted for the analysis of alkoxyacetic acids owing to significant chromatographic peak tailing and analyte carryover between injections (24). Also, the ethyl ester of the carboxylic acid was chosen for this study; other work using similar methodology (24) showed better compatibility with ethyl acetate extraction over esterification to the methyl ester (20). Possible cross esterification and production of a mixture of both methyl and ethyl esters was avoided.

It was the objective of this study to develop an effective and simple procedure to detect and measure the levels of 2-BAA in human urine samples. It was also a goal to have a validated (26–29) test in place for use in monitoring exposed individuals in future field studies by this laboratory. As briefly described previously, acid catalyzed esterification avoided many of the difficulties encountered using other derivatization methods. Capillary GC using a dimethyl polysiloxane phase (HP-1) column and detection by means of mass spectrometer (MS) proved to be specific for the analytes and eliminated interferences from the urine extract sample matrix for this analysis. Deuterated 2-butoxyacetic acid (d-2-BAA) was chosen as the internal standard for this chromatographic method. The various aspects of the development and validation of this test procedure will be discussed in detail.

## Experimental

### Chemicals and reagents

The 2-BAA used in this study was commercially available (Acros, Geel, Belgium). Deuterated 2-butoxyacetic acid (2-[($^2H_9$)butoxy]acetic acid) was synthesized and described previously by Brown et al. (12). All other reagents used were of analytical grade and are regularly available in a typical analytical laboratory.

### Instrumentation and chromatographic conditions

The chromatographic analysis was carried out using an Agilent Technologies model 6890 GC (Palo Alto, CA) 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: the helium carrier flow was 0.8 mL/min constant, and the injector port temperature was 220°C. The detector source temperature was 230°C with the quadrupole set at 150°C, which were the manufacturer's recommended settings. The column program was as follows: the initial temperature was 60°C and then increased to 150°C at a rate of 3°C/min. A post run of 230°C for 6 min was included with each run to remove artifact peaks. After the post temperature run, the GC was recycled back to the initial conditions and allowed to equilibrate, resulting in a complete GC run cycle of approximately 42 min. The mass selective detector was operated in electron impact mode with an electron energy of 70 eV, and selected ions were monitored at ion *m/z* 57 (2-BAA) and ion *m/z* 66 (deuterated 2-BAA) for quantitation. 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. The injection size of the final solution was 0.5 µL using splitless mode injection. The standard and sample vials were placed in the autosampler and injected. Signal data from the detector was not collected until 10 min after the injection to avoid solvent over load; the data signal was collected from 10 to 30 min after the injection.

### General urine sample preparation

Urine samples or 2-BAA-spiked urine samples were treated identically. A 4.0 mL portion of the urine was placed in a screw-capped tube and acidified with 30 µL of concentrated (12M) hydrochloric acid (approximately pH 1 to 2). A 0.5-mL aliquot of a 16 µg/mL of d-2-BAA internal standard solution was added. A 0.5 mL portion of deionized water for test samples or standard 2-BAA spiking solution was added. The urine sample was extracted four times with 5.0 mL of ethyl acetate using a vortex mixer for 1 min for each extraction. The ethyl acetate layers were combined and reduced in volume to 1 mL by evaporation using nitrogen sweep at room temperature. Each concentrated urine extract was treated with 2.0 mL of ethanol and 0.4 mL of concentrated sulfuric acid overnight (16 h) at 50°C in a heating block. After esterification, a 3.0-mL portion of methylene chlo-

ride was added to each sample, followed by the addition of 5.0 mL of deionized water. Extraction by use of a vortex mixer with 1 min time periods was performed. The methylene chloride layer was collected, and 5.0 mL of deionized water were added to the original esterified sample tube. This mixture was extracted three more times with 3.0 mL of methylene chloride. The extract solutions of methylene chloride were combined and dried with anhydrous magnesium sulfate. The methylene chloride solutions were reduced to 1 mL volume by nitrogen sweep evaporation at room temperature. The concentrated solutions were placed in crimp-capped vials for GC analysis.

### Standard sample preparation and recovery studies

2-BAA standards for calibration were prepared at the 0.1, 0.25, 1, 2, 5, 10, 20, 40, and 50 µg/mL equivalent levels in urine plus a blank 0 µg/mL level sample. Blank urine was spiked at the 1, 10, and 20 µg/mL equivalent 2-BAA levels for each experimental day of the primary recovery study. Three separate day or experimental periods were done for the primary recovery study. A secondary recovery study consisted of collecting urine from 20 "non-exposed" volunteers. Urine samples containing no added 2-BAA or the internal standard, urine samples with deuterated 2-BAA internal standard only, and urine samples spiked with 5 µg/mL equivalent 2-BAA level plus 2 µg/mL deuterated 2-BAA internal standard were prepared for this second recovery study.

### Quantitative calculation and the detection limit

Quantitative calculations were based on peak-area ratios of 2-BAA to its deuterated 2-BAA analog internal standard. At least three calibration curves, one at the beginning, one in the middle, and one at the end of each chromatographic batch run, using all the standards, were collected during the entire recovery study. Duplicate injections were performed for all spiked samples, and average values were calculated for the recovery data.

The limit of detection was calculated as three times the standard deviation of the noise level divided by the slope of the calibration curve (26). The average baseline level was determined for each batch run in chromatograms of the 0 µg/mL standard (in effect a blank chromatogram); at least three positions in the chromatogram were chosen near the retention time window for 2-BAA at *m/z* 57. The standard deviation from the baseline as height was determined for the noise level using 100 data points. Multiple 0 µg/mL 2-BAA level chromatograms were selected to produce and average standard deviation of the 100 data point noise instrumental level. The slope from the calibration curve using peak height ratios of all the standard solutions was used for this calculation.

## Results and Discussion

### Chromatographic conditions and method specificity

The chromatographic conditions developed for this test procedure appeared to be specific and had no major interferences. Typical single ion monitoring (SIM) chromatograms are shown in Figure 2. Chromatogram A displays two traces: *m/z* 57 for the detection of a 5 µg/mL 2-BAA spike in the urine sample and *m/z* 66 for the detection of a 2 µg/mL spike level of the deuterated 2-BAA internal standard. Chromatogram B shows the results of an unspiked urine sample; all background peaks were resolved from both analytes for their respective mass and retention times. As this method was designed with maximum sensitivity considered, the use of the analyte ion with maximum response (*m/z* 57) was necessary. This procedure also used a 50-m column to give maximum chromatographic specificity to the method. As can be seen in Figure 2, the only significant peaks not attributed to the analyte appear at retention time 14 to 16 min. The broad peak near 15 min is a response from the ethyl ester of sulfuric acid produced during the esterification step, which is at a high concentration and overloads the MS detector; it has been described in the literature previously by B'Hymer et al. (24). These peaks are readily separated and well resolved from the analyte peak and cause no problems for the method.

All of the unspiked urine samples showed no interfering peaks for 2-BAA ethyl ester, which included those from nonspiked samples and the chromatograms of urine samples from the "non-exposed" volunteers. However, three of the "non-exposed" volunteers did show low levels of 2-BAA (0.20 µg/mL and less for each). This was not completely unexpected as the parent 2-BE is so widely used in the general environment. Low level exposure among the general population would be expected as 2-BE is used extensively in paints, home cleaning products, and aerosols as described briefly in the Introduction of this manuscript. In the preliminary results of a field study on workplace exposure to 2-(2-methoxyethoxy)ethanol, a similar glycol ether used as a component in jet fuel formulation, urine levels of 8.4 µg/mL were detected for the corresponding biomarker, 2-(2-methoxyethoxy)acetic acid (24,30). Therefore, the "non-exposed" volunteers who had low levels of 2-BAA in their urine probably had been unknowingly exposed to a low amount of 2-BE from either at their home or work environment.

The use of a mass spectrometer for detection adds an obvious level of specificity to the procedure. The ion *m/z* 57

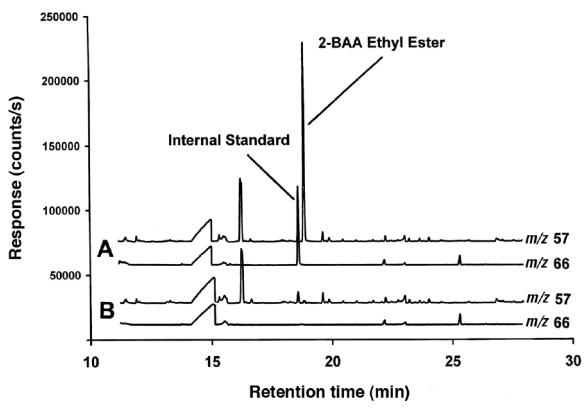


**Figure 2.** SIM chromatograms (*m/z* 57 and 66) of urine samples analyzed by the described procedure: a urine sample spiked at 5 µg/mL 2-BAA and 2 µg/mL of internal standard, the deuterated 2-BAA analog (A); and a nonspiked urine sample for comparison (B).

[$CH_3CH_2CH_2CH_2$-] was chosen for the calibration curve and in the quantitation because of its greater abundance as well as being a characteristic fragment for 2-BAA ethyl ester (see Figure 3). Ion *m/z* 66 [$CD_3CD_2CD_2CD_2$-] was monitored for the ethyl ester of the deuterated analog used as the internal standard for the same reasons. The use of other ions for qualification of peaks was considered; however, owing to the general lack of significant interferences, this was not considered necessary for this test procedure.

### Liquid–liquid extraction and selection of the internal standard

The liquid–liquid extraction (LLE) using ethyl acetate proved to be effective for this procedure in terms of the recovery of 2-BAA from spiked urine samples. Previous studies of the deuterated analog of 2-BAA showed an average extraction efficiency of 87% (range was 84 to 91%, $n$ = 3); this work used tert-butyldimethylsilane (TBDMS) derivatives of 2-BAA to measure the ethyl acetate extraction step directly against standard solutions (24). During early development work of the current method, a solid-phase extraction (SPE) procedure described by Brown et al. (12) was also evaluated; this procedure used Waters Oasis MAX cartridges (anion-exchange and reversed-phase mixed mode). The average recovery of d-2-BAA using a 6cc/500 mg bed Oasis Max cartridge was 12% (range was 9 to 14%, $n$ = 3) for 3 µg/mL spiked urine (24). This extraction yield was not adequate to gain maximum sensitivity for the detection of a biomarker. Although SPE generally offers a less labor intensive alternative for extraction, 2-BAA extraction was not suitable using the Oasis Max cartridge. Because it was the objective of this study to have the lowest detection limit and best sensitivity with a test method, ethyl acetate extraction of urine was the best approach. Another advantage of LLE over SPE is that bed capacity is not a consideration. Maximization of the extraction yield was the logical analytical approach for developing this method.

The use of the deuterated analog of 2-BAA as a procedural internal standard greatly improved accuracy and precision of the method. The deuterated analog would not be expected to be present in a worker exposed to glycol ethers. Generally, an internal standard compensates for changes in final sample solvent volume and dilution; however, the use of d-2-BAA as a procedural internal standard also compensates for minor differences in analyte extraction. Any extraction variability from an individual urine sample during extraction or in the esterification step should be compensated for by use of the deuterated analog; d-2-BAA would have the same solubility and extraction properties as 2-BAA analyte. Good calibration curves were obtained by using this internal standard; all curves generated within the 0.1 to 50 µg/mL 2-BAA range of this study had correlation coefficients of 0.99 or greater and y-intercepts near zero. Calibration curve slope drift was minimal (less than 4% was observed within any analytical sample batch run). Additionally, the use of the internal standard increased the precision of replicate chromatographic injections. Five replicated injections of the 5 µg/mL standard sample gave relative standard deviations (RSD) of peak area ratios ranging from 1.2% to 4.6% during the recovery studies.

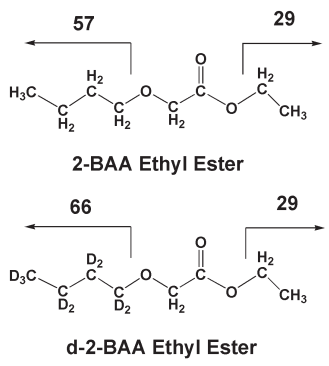


**Figure 3.** Ion *m/z* 57 was the major ion for the ethyl ester of 2-BAA, and it was monitored for generating the data for the method. Ion *m/z* 66 was the major ion for the deuterated analog used as the internal standard.

### Analyte recovery

A primary recovery study of urine spiked with 2-BAA was performed over thee separate batch runs to demonstrate the accuracy and precision of this test procedure. These data are shown in Table I; the average recovery was between 101% and 102% for the three 2-BAA spiked sample levels evaluated. For each run, the experimental trial consisted of three samples at the three concentration levels. The 1 µg/mL level had the highest average recovery at 102%, and the 10 and 20 µg/mL levels had

**Table I. Multilevel Recovery Study of 2-Butoxyacetic Acid***

| Spike Level (µg/mL) | Mean 2-BAA Recovered ($n$ = 9) (µg/mL ) | Average Percent Recovery | Standard Deviation (µg/mL) | % RSD |
|---|---|---|---|---|
| 1 | 1.02 | 102 | 0.027 | 2.6 |
| 10 | 10.1 | 101 | 0.21 | 2.1 |
| 20 | 20.1 | 101 | 0.23 | 1.1 |

* Notes: Three different spiked 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. The same HP-1 GC column was used for experimental batch trial 1 and 2; a second HP-1 column was used on trial run 3. % RSD = percent relative standard deviation.

**Table II. Recovery of 5 µg/mL 2-Butoxyacetic Acid Spikes from Urine Samples of 20 "Non-Exposed" Volunteers***

| Mean Recovery (µg/mL) | Average Percent Recovery | Lowest Value (µg/mL) | Highest Value (µg/mL) | % RSD |
|---|---|---|---|---|
| 5.01 | 100 | 4.92 | 5.32 | 2.8 |

* Notes: Three of the "non-exposed" volunteer samples had 0.2 µg/mL or less 2-BAA. These background levels of 2-BAA were subtracted out of the recovery results. % RSD = percent relative standard deviation.

an average recovery of 101%. These results are within statistical expectations for recovery and obviously displays no bias. The highest relative standard deviation was 2.6% for the 1 µg/mL spiked samples, which is more than acceptable for a biomarker assay test. This low variation on recovery is excellent, taking into consideration the two extraction steps and esterification, which confirms the high extraction efficiency of the LLE used.

A secondary recovery study used urine samples from 20 "non-exposed" volunteers and demonstrated further that the method was both accurate and precise (Table II). The 5 µg/mL spiked samples showed an average recovery of 100% and a relative standard deviation (RSD) of 2.8%. The lowest individual sample recovery was 4.92 µg/mL, and the highest was 5.32 µg/mL. As mentioned briefly before, three of the individual volunteer samples had an initial level of 2-BAA of 0.2 µg/mL or less. This level was subtracted from the total level detected in the 5 µg/mL spiked sample results shown in Table II. Because 2-BE is common in the modern environment, some "non-exposed" volunteers were likely exposed to low levels of this glycol ether. The second recovery study data indicates that differences in individual urine samples, that is slight matrix differences, do not cause any decrease in accuracy or precision for this test procedure.

**Method reproducibility, detection limit, and analyte stability**

Two different HP-1 capillary columns of different manufacturing lots were used during the recovery studies. This would indicate that the method is reproducible or robust, and the results obtained should be consistent with different HP-1 columns. The detection limit was found to range from 0.005 to 0.015 µg/mL 2-BAA equivalent levels in urine. The general condition of the two columns used and the day-to-day variation in detector noise and sensitivity accounted for this range. This detection limit is consistent with other alkoxyacetic acid analysis reported in the literature; the limit of detection (LOD) for urinary analysis methods for (2-methoxyethoxy)acetic acid has been reported at 0.01 to 0.05 µg/mL using GC–MS (24).

Aqueous stock standard solutions of 2-BAA used within 1 week appeared not to degrade; stability is implied for this period of analysis time. There was no evidence of 2-BAA ethyl ester degradation of sample solutions, although it would be expected that the internal standard should degrade at the same rate in the final chromatographic solution. Samples prepared from fortified urine and run within a week of initial preparation gave the same assay result, thus indicating short term analyte stability. This procedure should be considered accurate for the quantitation of free 2-BAA in human urine within the 0.1 to 50 µg/mL standard range. In the event that field samples would fall above this range, they could be diluted within the test range for analysis.

## Conclusion

An accurate and precise method has been developed and validated to monitor the level of the biomarker 2-BAA in human urine. Extraction of the analyte, followed by esterification and extraction of the 2-BAA ethyl ester, was necessary for analysis by GC with MS detection. Average recovery of 2-BAA fortified urine samples was between 100% to 102% of theory with relative standard deviations as high as 2.8% using urine samples at 2-BAA concentrations of 1, 5, 10, and 20 µg/mL. Standard curves generated linear response in the range of 0.1 to 50 µg/mL, with correlation coefficients of 0.99 and greater. The detection limit ranged between 0.005 to 0.015 µg/mL. This procedure has been demonstrated to be applicable for the quantitation of 2-BAA in urine.

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