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Synthesis, characterization, and use of 2-[($^2H_9$)butoxy]acetic acid and 2-(3-methylbutoxy)acetic acid as an internal standard and an instrument performance surrogate, respectively, for the gas chromatographic–mass spectrometric determination of 2-butoxyacetic acid, a human metabolite of 2-butoxyethanol ☆

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Abstract

2-[($^2H_9$)Butoxy]acetic acid and 2-(3-methylbutoxy)acetic acid were synthesized, mixed with 2-butoxyacetic acid, and separated by capillary gas chromatography on a fused-silica column with a length of 50 m, inside diameter of 0.200 mm, and a ''free fatty acid phase'' wall coating of 0.3 μm film. 2-[($^2H_9$)Butoxy]acetic acid, 2-butoxyacetic acid, and 2-(3-methylbutoxy)acetic acid were baseline resolved at retention times of 13.55, 13.78, and 15.20 min; 2-(3-methylbutoxy)acetic acid having a peak efficiency of 360 000. Mass spectrometric detection using selected ion monitoring at m/z 66, 57, and 71 showed linear analytical responses from 0.04 ng to at least 200 ng with a limit of detection of 0.04 ng for 2-butoxyacetic acid.

Keywords: 2-Butoxyacetic acid

1. Introduction

Butoxyethanol can be absorbed through the skin and long-term or repeated exposure may have effects on the hematopoietic system, resulting in blood disorders. 2-Butoxyacetic acid (BAA) is the major metabolite of butoxyethanol and is responsible for the hemolysis of red blood cells. Biological monitoring can be used to assess exposure through the skin. In 1990, the National Institute for Occupational Safety and Health (NIOSH) published *NIOSH Criteria for a Recommended Standard: Occupational Exposure to Ethylene Glycol Monobutyl Ether and Ethylene Glycol Monobutyl Ether Acetate*, and within that document reviewed the toxicity of butoxyethanol, its dermal absorption properties, and the previously published BAA biomonitoring methods with a recommendation for the Groesenken GC

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method [1]. All the methods reviewed by NIOSH, including Groesenken's, required derivatization techniques which add time, labor, and imprecision to analytical methods. For example, Groesenken's derivatization process required 24 h of freeze-drying and 3 h of reaction time for each sample. Historically, one way chemists avoided the time-consuming derivatization required in chromatography methods was to use liquid chromatography (LC) [2–4]. In LC, derivatization was more often used to enhance detector selectivity and sensitivity not chromatography efficiency [5]. One way to avoid derivatization in conventional reversed-phase liquid chromatography (RPLC) was by using ion-pairing reagents in the mobile phase [6]. Because LC methods lacked separation efficiency, they did not gain the acceptance of GC methods for the measurement of the alkoxyacetic acid metabolites in urine for biomonitoring. In 1994, Sakai et al. published another GC method that used analyte derivatization and flame ionization detection [7].

The purpose of this research was to develop a faster biomonitoring method for glycol ethers by eliminating analyte derivatization and using an isotopically diluted internal standard. In 1999, Tung-Sheng Shih et al. published a GC method that did not require derivatization of the analyte, because it used wall-coated open tubular (WCOT) capillary column technology with a stationary phase amenable to organic acids [8]. Components of the Shih et al. method were used in this work. In addition, solid-phase extraction (SPE) was used with custom synthesized 2-[($^2H_9$)butoxy]acetic acid (dBAA) as an internal standard. Previous BAA methods, including Groesenken's, used internal standards such as pentoxyacetic acid [9], 3-chloropropionic acid [10], or dichlorobenzene [5] to enhance accuracy and precision. The isotope dilution internal standard improved on these previous candidates [11].

Two compounds, 2-(3-methylbutoxy)acetic acid (MBAA) and 2-[($^2H_9$)butoxy]acetic acid, were prepared by the Pribish method previously used to make other alkoxyacetic acids, and their chromatographic and mass spectral properties were characterized. These compounds were studied as an internal standard and an instrument performance/recovery standard. MBAA was used as an instrument performance standard by adding it to each sample extract injected, and then monitoring the instrument's response to it during the study. MBAA was also used as a recovery standard by adding it to each sample extract injected, and calculating the internal standard recovery through the SPE step from its relative response to MBAA. This paper presents the GC–MS analysis of dBAA, MBAA, and BAA and characterizes the method for selectivity, sensitivity, linearity, range, and stability as an analytical platform for developing a SPE–GC–MS method for BAA in urine.

2. Experimental

2.1. Materials

BAA (98%+), methanol, (high-resolution gas chromatography grade), acetonitrile (Optima grade), and formic acid (99% reagent grade) were purchased from Acros through Fisher Scientific (Hanover Park, IL, USA). Sodium hydride (60% suspension in mineral oil), tetrahydrofuran (THF, anhydrous, unstabilized), bromoacetic acid (99+%), and 3-methyl-1-butanol (99+% anhydrous) were purchased from Aldrich (St Louis, MO, USA). ($^2H_9$)Butan-1-ol (98% atom% D) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The reactants and reaction solvents were used without further purification unless noted otherwise. The nuclear magnetic resonance (NMR) spectra were recorded of the products while in deuterated chloroform using tetramethylsilane as an internal standard on a Bruker AC 250-MHz instrument (Bruker BioSpin, Billericay, MA, USA). M-H-W Laboratories (Phoenix, AZ, USA) performed the elemental analysis.

2.2. Instrumentation

The GC analytical instrumentation consisted of a Hewlett-Packard (HP)-6890 gas chromatograph, HP-5973 mass spectrometer, and a HP-7673 automatic liquid sampler (Wilmington, DE, USA). Two types of HP-free fatty acid phase (HP–FFAP) GC columns were used (1) a 30 m×0.25 mm with 0.25 μm film, part no. 19091F-433 and (2) a 50 m×0.20 mm with 0.3 μm film, part no. 19091F-105. An HP 5 m× 0.250 mm I.D. deactivated retention gap (HP part no.

19091-60610) placed before the analytical column facilitated temperature focusing of the analytes.

2.3. Gas chromatography conditions

Standards in 2 μl of methanol/2% formic acid solutions were injected into the GC in the splitless mode at an injection port temperature of 250 °C. The carrier gas was ultra pure helium used in a constant flow mode of 1.0 ml/min, and a purge flow 50 ml/min with 2 min purge time. The oven temperature program had an initial isotherm of 80 °C for 2 min followed by a temperature ramp of 20 °C/min to 105 °C. The temperature was then raised to 240 °C at a rate of 70 °C/min, with a final isotherm of 15 min. The MS transfer line was held at 230 °C. The collection of MS data was delayed for 7 min in order to elute the solvent before activating the source filament.

2.4. 2-[($^2H_9$)butoxy]acetic acid synthesis

The custom synthesis of 2-[($^2H_9$)butoxy]acetic acid and 2-(3-methylbutoxy)acetic acid was done using the Pribish [12] method conducted according to the procedure of Zimmer et al. [13,14]. The reactions were carried out in an argon atmosphere. A 500-ml three-neck round bottom flask on a magnetic stirring hotplate, equipped with a dropping funnel and a reflux condenser, was charged with sodium hydride (12.07 g NaH suspension in mineral oil, corresponding to 7.24 g [320 mmol] of NaH; washed with three 20-ml portions of hexane to remove the oil) and 60 ml THF. It was heated to reflux. A solution of bromoacetic acid (9.02 g, 64.9 mmol, in 60 ml of THF) was placed in the dropping funnel and slowly added over 45 min. During this time hydrogen gas evolution was observed. Next, a solution of ($^2H_9$)butan-1-ol (5.0 g, 60.2 mmol, in 60 ml THF) was placed in the dropping funnel and slowly added over 30 min. Some more gas evolution was observed, and the suspension turned thick. After refluxing for 24 h, the solution was brought to room temperature, and 25 ml of water were added carefully to destroy the excess NaH, resulting in the evolution of hydrogen. Then, 30 ml of concentrated hydrochloric acid were added, and the mixture was diluted with 60 ml water. The two layers were separated, and the aqueous layer was extracted by using four portions of methylene chloride (total: 210 ml). The combined organic layers were washed once with 60 ml of saturated aqueous ammonium chloride solution, dried with anhydrous sodium sulfate, and concentrated in a rotary evaporator. The resulting yellowish oil was purified by distillation. The product collected was a clear and colorless oil occurring as a second fraction (b.p. 111–112 °C at 9 mmHg) after a very small first fraction.

The synthesis procedure had a product yield of 4.11 g (29.1 mmol, 48.4% yield). The molecular formula of $C_6H_3D_9O_3$ was consistent with the product's elemental analysis. The compound should theoretically have a molecular mass of 141.21 u and an exact mass of 141.1351 u. The final product was collected over a boiling point range of 125– 130 °C (~ 15 mmHg). In the elemental analysis, percent carbon was calculated at 51.03%, and 51.19% was found. The percent H (all H and D were expressed as H) was calculated at 8.50% and was found at 8.33%.

NMR ^{1}H analysis found δ=4.13 (s 2H) and δ= 10.3–10.7 (bs 1H). NMR ^{13}C analysis found δ= 67.59 and 175.45. The signals of the carbon atoms bound to deuterium atoms (quintets and septets) did not rise above the background noise.

2.5. 2-(3-Methylbutoxy)acetic acid synthesis

A 250-ml three-neck round bottom flask on a magnetic stirring hotplate, equipped with a dropping funnel and a reflux condenser, was charged with NaH (4.960 g suspension in mineral oil, corresponding to 2.976 g [124 mmol] NaH, and washed with three 8-ml portions of hexane) and 25 ml THF. It was heated to reflux. A solution of bromoacetic acid (3.692 g, 26.6 mmol) in 25 ml THF was placed in the dropping funnel and slowly added over 45 min. During this time hydrogen gas evolution was observed. Next, a solution of 2.692 3-methylbutanol (2.178 g, 24.7 mmol) in 25 ml THF was placed in the dropping funnel and slowly added over 30 min. More gas evolution was observed and the suspension turned thick. After refluxing the suspension for 24 h, it was brought to room temperature and 10-ml of water was added carefully to destroy the excess NaH, accompanied by an evolution of hydrogen gas. Then, 12 ml of concentrated hydrochloric acid was

added, and the mixture was diluted with 25 ml of water. The two layers were separated and the aqueous layer was extracted by using four portions of methylene chloride (total 90 ml). The combined organic layers were washed with 25 ml of saturated aqueous ammonium chloride solution, dried with anhydrous sodium sulfate, and concentrated on a rotary evaporator. The resulting, lightly yellowish oil (3.95 g) was purified by distillation. The product collected was a clear, colorless oil, occurring as a second fraction (b.p. range 118–122 °C 15 mmHg) after a very small fraction.

The synthesis procedure had a product yield of 2.23 g (15.3 mmol, 61.8% of theoretical). The molecular formula of $C_7H_{14}O_3$ was consistent with the product's elemental analysis. High-resolution mass spectrometry (HRMS) of the product detected a molecular ion with a m/z ratio of 147.0979 as compared to its theoretical exact mass of 146.0943 u. The product was collected over a boiling point range of 118–122 °C (~15 mmHg). In the elemental analysis, the percent carbon in the formula was calculated at 57.51%, and 56.63% was found. The percent H in the formula was calculated at 9.65% and 9.21% was found.

High-resolution mass spectrometry gave ions at m/z=147 (M+1), 101, 87, 71, 69, 61, 55, and 43 (100%).

NMR ^{1}H analysis found δ=0.90, 0.93 (s,s 6H); δ=1.53 (m 2H); δ=1.71 (m 1H); δ=3.59 (t 2H); δ=4.13 (s 2H); and δ=11.0–11.4 (bs 1H). NMR ^{13}C analysis found δ=22.36, 22.45, 24.81, 38.10, 67.60, 70.42, and 175.67.

2.6. Standard solutions

Standard stock solutions of dBAA, BAA, and MBAA were each prepared at 100 mg/100 ml concentration in acetonitrile, and stored at 5 °C. Calibration solutions were prepared as needed by dilution from the stock solutions. The calibration solutions were prepared from the standard stock solution by dilution with acetonitrile; however 2% formic acid was included. Formic acid was added to ensure that the alkoxyacetic acid standards were protonated before GC analysis. The standard stock solutions produced a stable response (peak area/ng) throughout the 1-year study indicating no sample degradation.

2.7. Experiment 1: GC–MS of BAA, dBAA, and MBAA

The initial chromatographic test was done by sequentially injecting five samples: (1) a solvent blank, (2) a 100 mg/l BAA solution, (3) a 100 mg/l dBAA solution, (4) a 100 mg/l MBAA solution, and (5) a mixture of 33 mg/l BAA, 33 mg/l dBAA, and 33 mg/l MBAA, using the instrument's mass scanning mode from 50 m/z to 550 m/z. The resulting chromatograms and mass spectra were examined.

2.8. Experiment 2: target and qualifying ion selection

To determine the best target and qualifying ion to monitor for analytical work, a 10 mg/l BAA, dBAA, and MBAA calibration solution was analyzed three times using three injections by GC–MS in the selected ion mode (SIM). The detector output signals were recorded at the instrument m/z settings of 57.15, 56.15, 60.15, 73.1, and 87.1 (fragments found in BAA MS spectrum) during the first GC analysis, at the instrument m/z settings of 66.25, 64.15, 62.15, 42.15, and 34.0 (fragments found in dBAA MS spectrum) during the second GC analysis; and at the instrument m/z settings of 71.2, 70.2, 69.2, 55.2 and 41.2 (fragments found in MBAA MS spectrum) during the third analysis. Thus, each analysis produced five chromatograms, one from each ion monitored. The signal output of each chromatogram was measured for background level (the zero offset), the standard deviation of the background signal (N), maximum signal output (S), and the maximum signal to background ratio (S/N). The ion with the highest S/N ratio was chosen as the target ion.

2.9. Experiment 3: BAA and BAA/dBAA detector linearity and sensitivities

The BAA calibration solutions varied in BAA concentration, however the dBAA and MBAA concentrations in each of the calibration solutions were prepared at the same level of 10 mg/l. The 21 calibration solutions varied in targeted BAA con-

centrations of 0.01000, 0.02000, 0.04000, 0.06000, 0.08000, 0.1000, 0.2000, 0.4000, 0.6000, 0.8000, 1.000, 2.000, 4.000, 6.000, 8.000, 10.00, 20.00, 40.00, 60.00, 80.00, and 100.0 mg/l. The four significant figure numbers indicate that all mass and volume measurements were made to an accuracy and precision of at least four significant figures when preparing these solutions.

2.10. Experiment 4: analyte carryover

The calibration solutions in experiment 3 were randomly analyzed with blanks between each calibration solution injection. The blank solutions were measured for BAA, dBAA, or MBAA artifacts from the previous injections.

2.11. Experiment 5: evaluating MBAA and dBAA as internal standards

A 10-mg/l solution of BAA, dBAA, and MBAA was injected 90 times with 2-μl aliquots and syringe washing between injections. The peak heights of each component were tabulated and statistically analyzed for accuracy and precision.

2.12. Experiment 6: MBAA as an instrumental performance surrogate

Standard stock solution MBAA at 1.0 mg/ml was diluted to 110 p.p.m. One hundred microliters of the 110 p.p.m. MBAA solution was added to 1.000 ml of the urine extract to make a sample with a nominal concentration of 10 mg/l. Starting with a new column, the MBAA spiked urine extract was injected 128 times using 2-μl aliquots and syringe washing between injections. The chromatograms were integrated for peak area and height using selective ion monitoring (SIM) mode at the MBAA target ion m/z.

3. Results

3.1. GC mass spectrometry of calibration solutions

The 30-m FFAP capillary column retained dBAA, BAA, and MBAA for a t_R of 9.50, 9.72, and 11.10 min, respectively. The 60-m FFAP capillary column retained dBAA, BAA, and MBAA for a t_R of 13.55, 13.78, and 15.20 min. Fig. 1 shows the resulting chromatograms from injecting the calibration solution mixture under the conditions of two different column lengths. In both systems all three components were separated with the 2-butoxyacetic acid being resolved from its corresponding dBAA. There appears to be a small shoulder peak with dBAA in the 60-m chromatogram. Fig. 2 shows the mass spectrum of components taken at the center of their respective chromatographic peaks. From most abundant fragment to least, the BAA's spectrum showed fragment ions at m/z of 57, 56, 60, 73, and 87; dBAA's spectrum showed fragment ions at m/z of 66, 64, 62, 96, and 82; and MBAA's showed fragment ions at m/z of 71, 70, 69, 55, and 101.

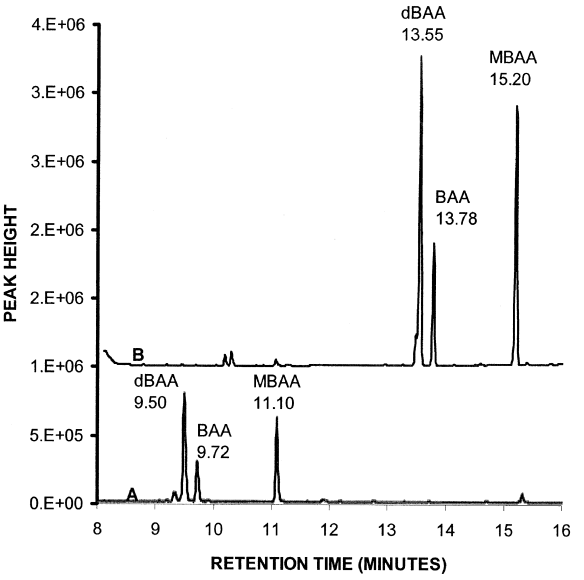

Fig. 1. Chromatograms of dBAA, BAA, and MBAA in acetonitrile/2% formic acid from two different columns, (A) a 30 m, 0.25 mm, 0.25 μm HP–FFAP, and (B with response offset of 1E+06 units) 50 m, 0.20 mm, 0.3 μm HP–FFAP. Helium carrier had a constant flow of 1.0 ml/min, and the detection was done using MS–TIC.

The initial temperature of the gas chromatograph oven was a critical variable in separation performance. In an experiment varying the initial column temperatures from 55 to 105 °C in increments of 5 °C, a temperature of 80 °C was found to provide the optimum sample introduction temperature. Below

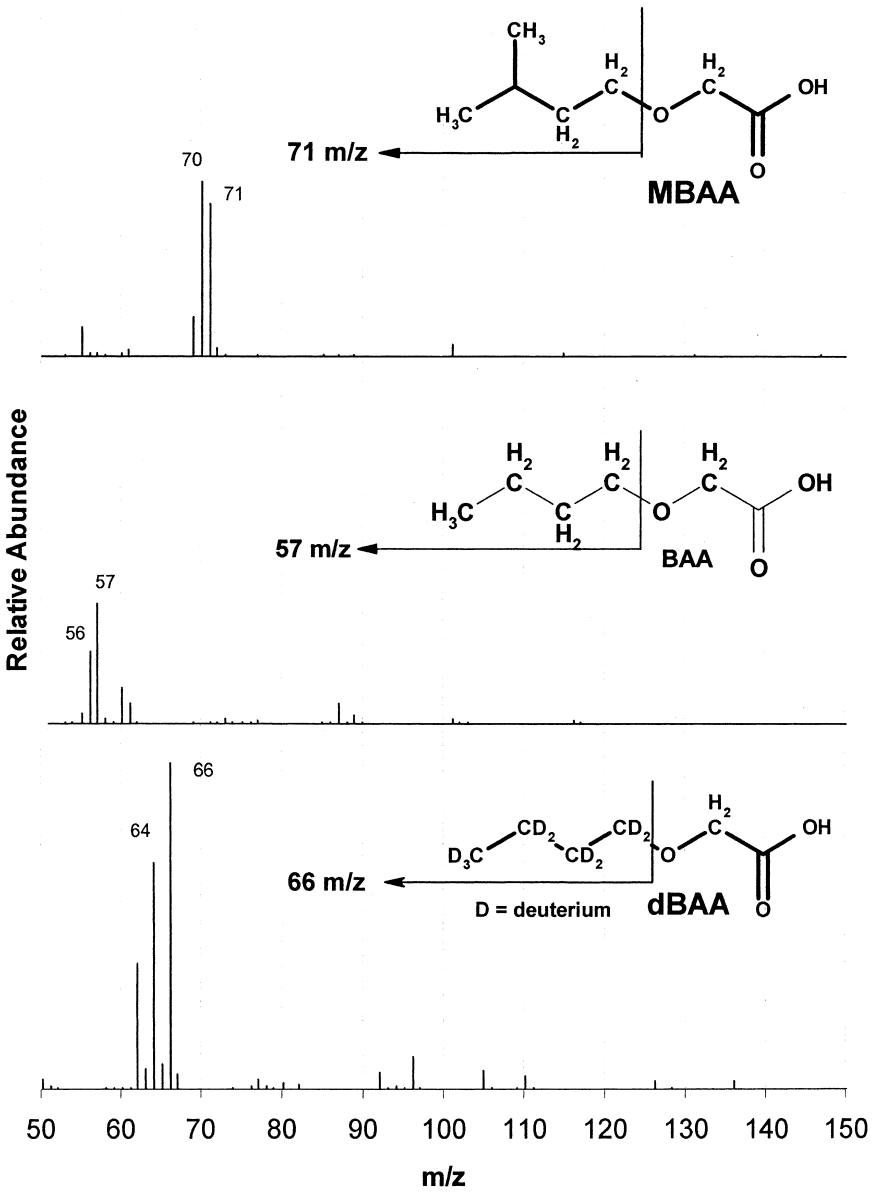

Fig. 2. The mass spectra of dBAA (perdeuterobutoxyacetic acid), BAA (2-butoxyacetic acid), and MBAA (2-methylbutoxyacetic acid) are presented. The most abundant fragment is the alkyl moiety of the molecule in each case of fragmentation.

a temperature of 80 °C, the column irreversibly deteriorated and above 80 °C, the chromatography efficiency dropped significantly. It appears that if the solvent vapors from the injection port condensed on the column at a temperature and pressure below their boiling points, then band broadening and rapid column deterioration occurred. However, an optimum initial condition occurred at a column temperature and pressure that allowed for the passage of solvent and formic acid in gas phase through the column, while condensing and focusing the analyte and its urine extract matrix on the front of the column. A highly deactivated 5-m fused-silica precolumn helped to facilitate sample focusing.

3.2. Target and qualifying ion selection

Of the five most abundant ions found in the mass spectrum of each compound, the BAA ions of m/z 57 and 60, the dBAA ions of m/z 66 and 96, and the MBAA ions of m/z 70 and 101 provided the highest signal-to-noise and signal-to-background ratios and were used for SIM. From each compound's pair of selected ions, one was used as the target and one as the qualifying ion. The alkyl fragments were the most abundant and thus used as the target ion for quantification. A non-alkyl fragment of each molecule was chosen as the qualifying ion, because a secondary alkyl fragment is also common to many other compounds and does not confirm the identity of the alkoxyacetic acid.

3.3. Characterizing BAA and BAA/dBAA detector linearity and sensitivities

In Fig. 3, the data-set no. 1 points shows the calibration response for BAA as peak height for over four orders of magnitude from 0.01 to 100 mg/l, (0.02 to 200 ng on column), and linear regression analysis of data point set no. 1 resulted in a linear equation of $y=5500x+26$ with $r^2=0.99$ and $Se_y=28\,860$ where y is in arbitrary units of peak height, x is in nanogram units, r^2 is the linear regression correlation coefficient, and Se_y is the linear regression standard error about the Y-axis in peak height units. The data-set no. 2 points for dBAA shows a constant response for the internal standards that have the linear regression equation of $y=57.53x+104\,048$. The dBAA linear regression analysis was not a calibration curve, but a check on consistency. The average response for the internal standard was 104 048 with a slight positive drift of 2% over 40 injections. From the dBAA data, the instrumental RSD was calculated from 100 Se_y/intercept(b) at 13%. Se_y/intercept(b) was used instead of RSD divided by the average in order to remove variability components due to drift. The no. 3 set of data points is the response ratio of BAA to dBAA peak heights with BAA varying from 0.02 to 200 ng and dBAA held a constant 20 ng per injection. Linear regression analysis of the data-set no. 3 points, BAA/dBAA, resulted in an equation of $y=0.04895x+0.0208$ with $r^2=0.998$ and $Se_y=0.123$ where y and Se_y are the unit-less ratio of BAA peak heights to dBAA peak heights.

The data-set no. 4 points in Fig. 3B, BAA/dBAA, shows the calibration data for only 0.02 to 0.18 ng, and the linear regression analysis resulted in an equation of $y=0.06841x+0.0002253$ with $r^2=0.91$ and $Se_y=0.0012$. The no. 4 set of data points were used to calculate the instrumental limit of detection (LOD), defined as 3*(Se_y)/slope and equal to 0.020 mg/l (0.040 ng). However, the data point at 0.02 ng was suspected to be an outlier below the LOD, and was not used in the estimate of the LOD. Since a method's precision is generally a non-linear function over several orders of magnitude of concentration, the regression analysis of this method's precision near the LOD was kept to data points within one order of magnitude above the suspected LOD.

3.4. Analyte carryover

All three standards were found in the blank solvent injected immediately after a calibration injection, and the peak height of the amount found of standard in each blank (y) was a linear function of the peak height response of the calibration solution injected before that blank. For BAA, the linear regression analysis of this data resulted in an equation of $y=0.08x+1164$ with $r^2=0.99$ and $Se_y=2036$, x is in units of peak height response of a calibration injection, and y is in units of peak height response of the blank following calibration injection. The blank response averaged a 8% carryover from its previous calibration injection. These results indicated the need for multiple syringe washings between each injection

3.5. Comparing MBAA and dBAA as internal standards for BAA

The results of injecting a 10 mg/l MBAA, 5 mg/l BAA, and a 10 mg/l dBAA solution 90 times are summarized in Table 1. The SIM of BAA used ion m/z ratio instrumental settings at 57.15 and 60.15; MBAA, 71.15 and 101.15; and dBAA, 66.3 and 96.3. Linear regression analysis of the peak heights over time showed a negative response drift due to column efficiency deterioration. However, the ratio of BAA or MBAA response to internal standard peak

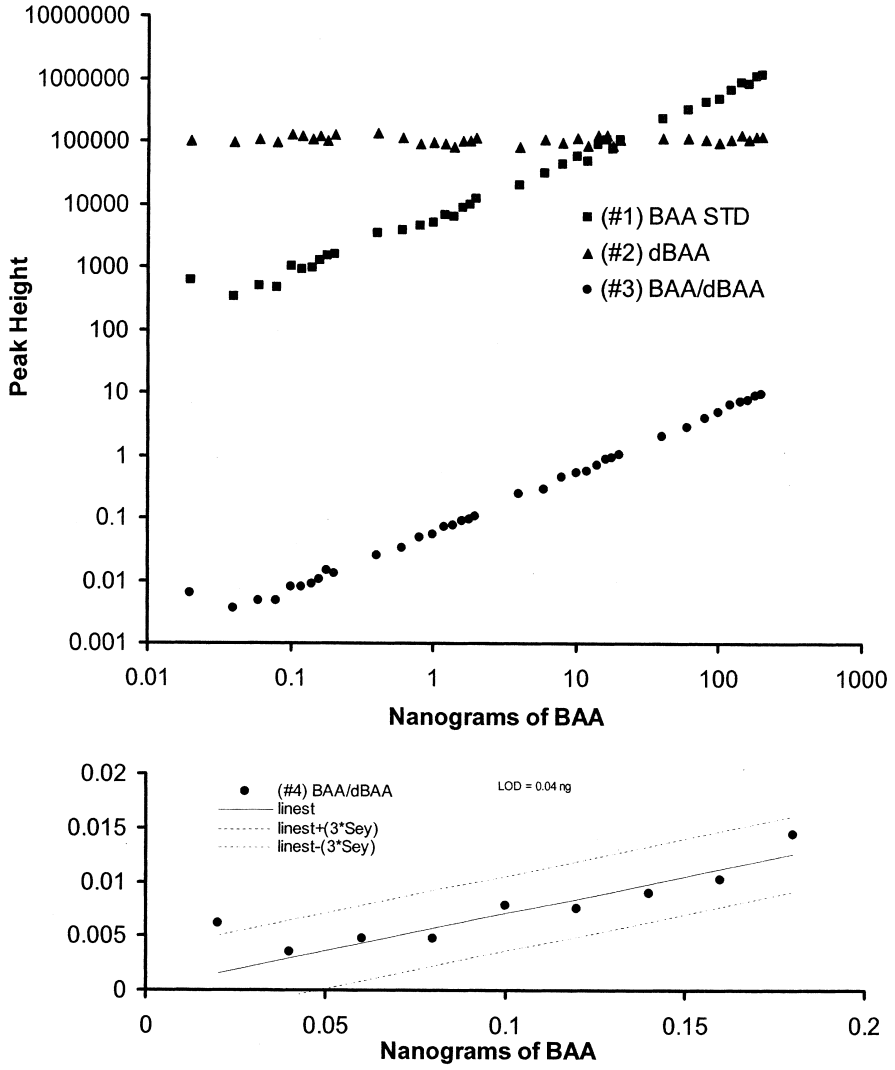

Fig. 3. Calibration plots of (1) BAA standards response, (2) the 10 p.p.m. dBAA response, and (3) the response ratio of BAA/dBAA and (4) BAA/dBAA response ratio for lower range of standards from 0.020 to 0.18 ng with linear regression line and $\pm 3*Se_y$. The LOD is 0.04 ng. The injection volume was 2 μl.

response remained constant over time, because the deterioration rate in chromatographic efficiency was the same for each of the three components. The RSD of BAA was 8.3% as estimated from the $100*Se_y$/intercept. The use of an internal standard reduced the variability of BAA from 8.3% to 5.4%. The drift error and instrumental imprecision of measuring BAA was reduced equally when dBAA and MBAA was examined as an internal standard.

3.6. MBAA column efficiency

Fig. 4 shows the response of MBAA at 10 mg/l in urine extract for 128 consecutive 2 μl injections. The data was integrated for both peak height and area. Base width of each peak was calculated from (2*area)/height from the equation for the area of a triangle. Peak efficiency of each peak was calculated as $N=16*(t_R/W_b)^2$ (t_R=retention time, W_b=peak

Table 1
The statistical results from injecting a 10 mg/l calibration solution of MBAA, dBAA, and BAA 90 times in sequence

	MBAA	BAA	dBAA	BAA/dBAA	BAA/MBAA
m/z	71.2	57.2	66.3	57.2/66.3	57.2/71.2
N	90	90	90	90	90
Average signal	24 492	5356	22 968	0.235	0.192
SD	2703	446	2843	0.019	0.010
%RSD	11.03	8.33	12.38	8.095	5.405
Regression data					
Slope	−34.78	−3.94	−41.34	0.000	0.000
r^2	0.11	0.05	0.14	0.117	0.112
Se_y	2560	437	2645	0.018	0.010
Intercept	26 075	5536	24 849	0.224	0.186
Se_y/intercept×100	9.82	7.89	10.64	8.038	5.288

The signal was measured as the peak height of each component.

width at base] and plotted dependent on injection sequence number. Peak efficiency was analyzed by linear regression which provided the equation, $Y=363340-574$*injection no. There was deterioration in column performance over the 90 injections that started at $N=363340$ and ended with $N=297330$, an 18% drop in performance. Injection to injection variance was 15% RSD. This variability and drift made it difficult to observe column performance by any one injection. Therefore, four quality control techniques were compared for their ability to predict the column deterioration rate and replacement time that was determined from post data collection linear regression analysis: (1) a cumulative mean, (2) a cumulative sum, (3) exponentially weighted moving average, and (4) moving average chart. A combination of the cumulative mean and moving average was chosen because they best fit the linear regression line, and can be used to determine the injection number at which the column performance reached its lower limit by extrapolation. The quality control (QC) chart starts by using a cumulative mean mode up to 30 samples and then continues as a moving average after sample 30. The lower limit of acceptance in column performance was determined by the need of method resolution. The overall value of Se_y was close to the SD of the first six observations, an the initial performance of a column was estimated from the first six observations in terms of average *N* and SD of *N*. These initial terms could be used to set the lower limit of column efficiency on a QC chart of cumulative mean, up to 30 observations, and then one could use a moving average chart.

Fig. 5 shows the instrumental response, MBAA peak area/ng, for the same 128 injections. Area/ng is independent of column efficiency. Again the variability from injection to injection is relatively large, 26% RSD, as calculated from a linear regression equation of $Y=4954-1.04$*injection no. The slope close to zero indicated that response deterioration was negligible, unlike the column efficiency. The lower limit can be set by the sensitivity needs of the method considering variability trends in instrument performance. A QC chart technique that best fit the deterioration of the detector from injection to injection as compared to post data linear regression analysis was, again, a combination of cumulative mean up to injection 30 and then a moving average of $n=30$. These results show how much the instrumental sensitivity varies from day to day, and injection to injection, and demonstrates the need for internal standards with each injection.

4. Discussion

4.1. GC mass spectrometry of calibration solutions

The deuterated compound confirmed that the BAA molecule does cleave at the alkyl ether linkage upon electron impact. Analogously, MBAA provided a unique fragment from its alkyl moiety. Three com-

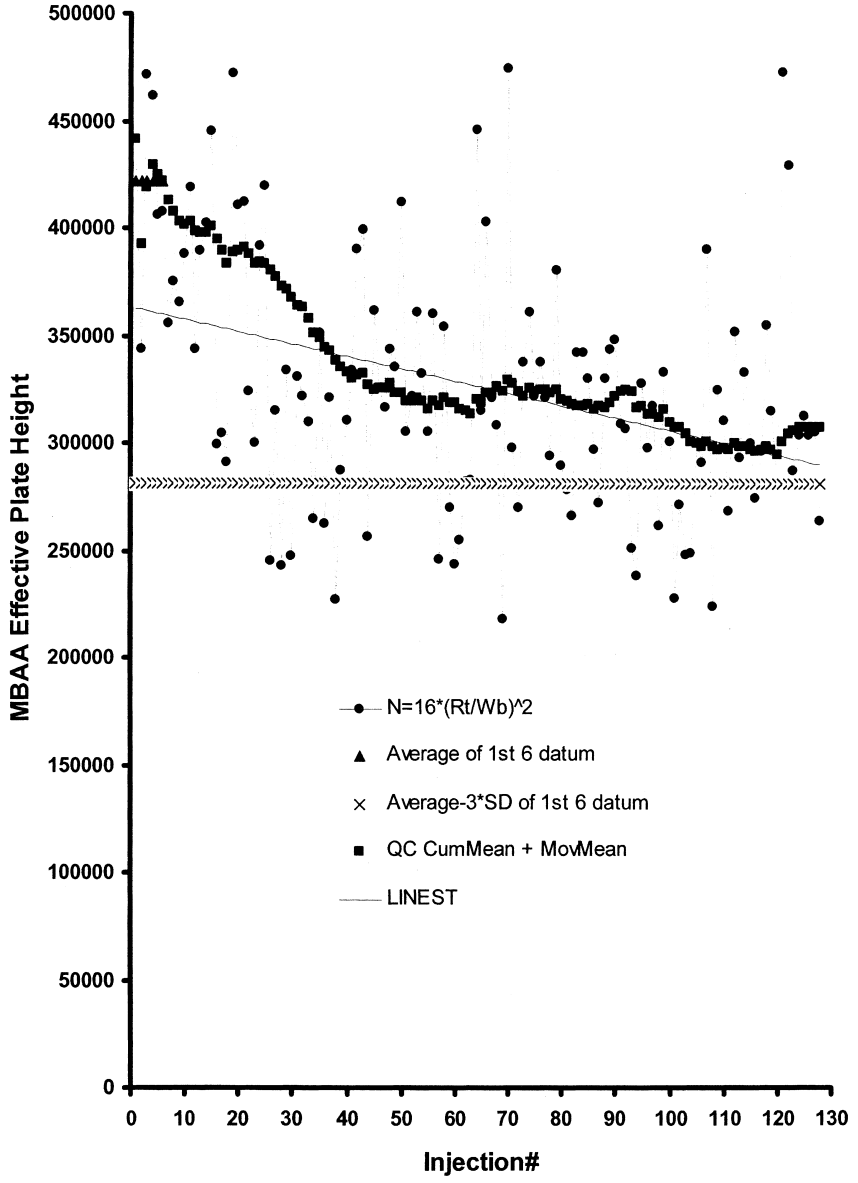

Fig. 4. MBAA used to measure chromatographic efficiency in terms of effective plate height examined on a QC chart.

mon fragments at m/z 46, 45, 44 were found in each standard's mass spectrum suggesting that these fragments were from the acetic acid moiety of the molecules. A fragment with the m/z of 60 commonly associated with acetic acid was found in the BAA and MBAA spectrum but not the dBAA spectrum. Replacing the hydrogen atoms on the butyl moiety of BAA with deuterium reduced the compound's retention time. BAA and dBAA were chromatographically baseline resolved. Adding a methyl group to BAA significantly increased its retention.

The nine-deuterium dBAA molecule was chosen for synthesis as an isotopically diluted internal standard, because deuterobutanol was the most convenient and economical isotopic reactant available. dBAA and BAA were resolved chromatographically,

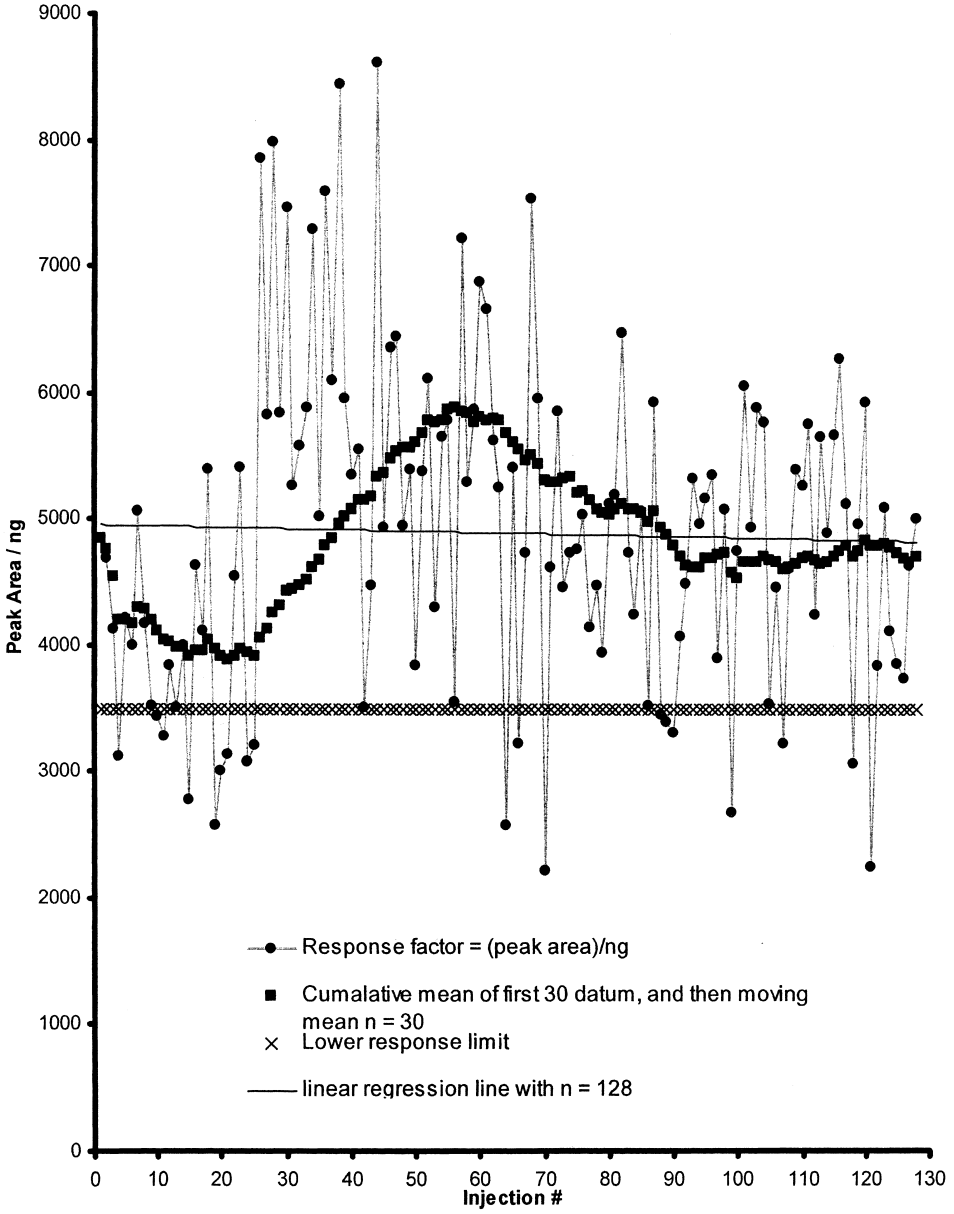

Fig. 5. MBAA instrumental response factor in terms of peak area per ng was used to monitor the quality of instrument's sensitivity.

indicating the isotopically diluted internal standard had different properties than its analyte. The ideal isotopically diluted internal standard would have the same physical and chemical properties as the analyte, in order to encounter the same components of variation through the sample preparation and chromatography processes eventually being resolved by mass spectrometry at the point of detection. The MS detector was capable of resolving an isotopically diluted internal standard differing in only 2 m/z. Thus, another isotopically diluted internal standard that differed by only 2 u may have mimicked the analyte's properties better and may have improved the method precision and accuracy even more.

4.2. Target and qualifying ion selection

The MS alkyl fragments of BAA, dBAA, and MBAA at m/z of 57, 66, and 71, respectively, were selected as target ions because they provided best S/N having maximum relative abundance. These alkyl fragments are common to many compounds, so the qualifying ion of m/z 60 from the molecule's acetic acid moiety was selected to confirm the identity of the BAA. The signal at m/z 60 was weak in the MBAA and dBAA mass spectra, so the stronger m/z 101 signal was used for MBAA and the stronger m/z 96 signal for dBAA. The qualifying ion for MBAA at m/z 101 resulted from the loss of the carboxylic acid moiety (–COOH) m/z 45 from the parent molecule, i.e. 146–45=101. Similarly in the dBAA case, the loss of the m/z 45 –COOH fragment from the parent molecule resulted in the qualifying ion of m/z 96.

4.3. BAA and BAA/dBAA detector linearity and sensitivity

This GC–MS analytical system resulted in an LOD of 0.04 ng (20 p.p.b.), a linear response over four orders of magnitude using SIM.

4.4. Analyte carryover

Analyte carryover was an issue with this method that did not appear when the analyte was derivatized and separated on an HP-1 column. The exact retention mechanism and retention sites (column, injection port, or syringe) causing the carryover were not diagnosed, however the problem was solved by 15 automated syringe washes after each injection with a basic solution (2% NH_4OH, 50% methanol, and 48% water) followed by 15 syringe washes with methanol. The syringe washes were programmed into the method and the wash was conducted during the chromatographic run adding no extra time to the method. If the methanol rinse had not been used, corrosion and freezing of the syringe barrel would occur. The use of a 5-μl syringe minimized the total volume of syringe wash volume needed during a batch run allowing the instrument to run unattended. While this syringe cleaning was excessive compared to traditional derivatization methods, it still seemed to be an advantage over the additional manual labor, imprecision, and time associated with analyte derivatization procedure.

4.5. MBAA as surrogate and dBAA as internal standards

When studied as an internal standard, both MBAA and dBAA reduced errors in measuring BAA. dBAA was used as internal standard on the hypothesis that it would perform better than MBAA during future extraction processes. MBAA was used as an instrument performance surrogate and recovery standard.

4.6. MBAA column efficiency

Peak height is a function of both column efficiency and detector sensitivity. Peak area is a function of detector sensitivity alone, independent of column efficiency. Therefore, a peak area QC chart was used to monitor instrument response, while peak height QC chart was used to monitor column performance. MBAA was used as an instrument performance surrogate by adding the compound at 10 p.p.m. to each sample injected into the GC–MS to track instrument and column performance.

Monitoring the chromatographic peak MBAA provided QC charts on the GC column performance throughout the study in terms column plates efficiency. From injection to injection: the analytes, BAA and dBAA, never see the same instrumental conditions twice because the column deteriorates, the detectors foul, and the instrumental parameters vary slightly with each injection. The column deterioration is slow, but progressive. Formic acid and urine extract accelerated the deterioration. The deterioration of this polar FFAP column by these free organic acids seems faster than experienced using a non-polar column with non-polar compounds; however, the cost and labor of replacing columns still seems less than the cost and labor of derivatization of free acid to non-polar derivatives. Moreover, derivatization adds a component of variation to the method precision. For column deterioration QC, the lower limit of efficiency can be set from statistics on the column's first six injections. By setting an efficiency lower limit and monitoring column efficiency, the

time to replace the column can be extrapolated from the rate of deterioration.

Monitoring peak area of MBAA provided detector and instrument QC. Using a QC chart, having both cumulative mean and moving average, provided data smoothing of injection-to-injection variability. The peak area chart showed that overall sensitivity of the GC–MS system remained constant while the GC column efficiency dropped. This chart showed an interesting sinusoidal drift in sensitivity of the GC–MS system during the experiment.

One of the advantages of incorporating a surrogate compound throughout a study is the QA documentation it provides. Some disadvantages of this approach are the added labor of adding 100 μl to each injection vial, the dilution of extract by the surrogate solution, the added data acquisition, and the time and labor of data processing. MBAA could also be used as a recovery standard for dBAA and BAA extraction from urine.

This GC–MS system was used in the further development of a solid-phase extraction method for biological monitoring of these compounds in urine.

5. Conclusion

The derivatization of alkoxyacetic acids can be avoided in GC–MS analytical methods by using a FFAP deactivated stationary phase in the WCOT capillary column. The direct injection of alkoxyacetic acids with formic acid resulted in a greater potential for sample to sample analyte carryover error and did accelerate column deterioration when compared to the injection of alkoxyacetic acids derivatives without formic acid. However, the chromatographic efficiency for these free acids was excellent and it provided symmetric peaks. The method's sensitivity and precision resulted in an LOD of 0.04 ng. The linear dynamic range of analysis extended over at least four orders of magnitude to 200 ng. MBAA can be used as a recovery standard and instrumental performance check during analysis. BAA, dBAA, and MBAA provided unique MS spectra for peak identification and confirmation. The internal standard, dBAA, significantly improved method accuracy and sensitivity. BAA and dBAA were chromatographically resolved.

Fig. 4 shows that, if an external calibration curve was prepared from the first chromatograms on this chart and if the last chromatogram was a sample containing analyte quantified from the external calibration curve, then there would have been an 18% error in measurement. The use of an internal standard would have normalized that error. Fig. 5 shows the sensitivity of the GC–MS system can vary as much as 50% from a low at sample no. 20 to a high at sample no. 55. Consequently, the method LOD can vary 50% between these samples. If the data report expressed the non-detection of an analyte as ''<LOD'', then there may be an error of 50% in reporting these samples. The use of an internal recovery standard could be used to monitor instrument sensitivity and determine an individual sample LOD for more accurate reporting.

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