Gas Chromatography Analysis of Urinary Alkoxyacetic Acids as Biomarkers of Exposure to Aliphatic Alkyl Ethers

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Abstract
Analysis of alkoxyacetic acids has received considerable research interest in toxicology because these compounds have been reported as metabolites and biomarkers of exposure to widely used industrial chemicals such as alkyl-substituted ethylene glycols and other aliphatic ethers. This paper describes an improved method for the determination of methoxyacetic acid (MAA), ethoxyacetic acid (EAA), and butoxyacetic acid (BAA) in rat urine. Solid-phase extraction with Bakerbond® C18 bonded silica cartridges was successfully employed to isolate the acids from rat urine. The acids were then converted to methyl esters with diazomethane derivatization and analyzed using a gas chromatograph (GC) equipped with a mass spectrometer (MS) and a GC with flame ionization detector (FID). Employing GC-MS under selected ion monitoring detection, the lowest detection concentrations for MAA, EAA, and BAA were determined to be from 2 to 4 ng/mL urine in 1 mL of sample size. This method is 5 to 10 times more sensitive than that using GC-FID. The method described here is superior to the existing ones reported in the literature in that it employs an easy sample treatment procedure and gives much higher recoveries, making it suitable for routine assays. The utility of this new method was demonstrated in a toxicology study of aliphatic alkyl ethers.

Introduction
In our recent toxicity studies of mono- and di-oxygenated alkyl ethers as potential novel diesel fuel additives (1,2), we observed that 1,6-dimethoxyhexane (DMH) produced testicular atrophy, thymic atrophy, and anemia in rats. An examination of the chemical structures of these oxygenated compounds revealed that DMH bears structural similarity to that of industrial chemical ethylene glycol monomethyl ether. A review of the literature led us to speculate that the toxicological effect of DMH may have been mediated through the formation of the toxic metabolite methoxyacetic acid (MAA) (3,4). MAA and its analogs ethoxyacetic acid (EAA) and butoxyacetic acid (BAA) have been reported as the putative toxic metabolites for the toxicity of respective ethylene glycol monomethyl, monooethyl, and monobutyl ether (5,6). In order to fully characterize the toxic effects of DMH and related alkyl ethers, we sought to develop a good analytical method to quantify MAA levels in urine.

In the literature, a number of methods have been reported for the determination of urinary MAA or its EAA and BAA analogs (7-21). These methods involve extraction of the alkoxyacetic acids with liquid-liquid extraction (LLE) or XAD resin, followed by derivatization of extracts prior to gas chromatographic (GC) analysis. It was found that recoveries of MAA and EAA by LLE methods were poor, probably because of the hydrophilic character of these compounds. Further, it was found that solid-phase extraction (SPE) with columns of XAD resin (20,21) were equally undesirable as the manual packing of columns and repeated solvent rinses required before use made the process highly laborious. In the current paper, we demonstrate that the commercially available Bakerbond® C18 bonded silica cartridge can be an effective absorbent for extraction of the alkoxyacetic acids. Use of C18 cartridges for SPE is simple and convenient, providing the basis for high throughput sample treatment for GC analysis of urinary MAA, EAA, and BAA. As a whole method, the alkoxyacetic acid extract from urine sample was derivatized with diazomethane and determined with GC with a mass spectrometer (MS) or flame ionization detector (FID).

Experimental

Chemicals
MAA (98%), EAA (98%, both from Aldrich, St. Louis, MO), and BAA (99%, Acros, New Jersey) were used without further purification. Calibration standard solutions were prepared by
dissolving appropriate amounts of MAA, EAA, and BAA in isopropanol to make the final concentrations at 2.00, 1.00, and 0.50 mg/mL, respectively. Standard solutions used to spike urine samples were prepared in a similar manner, except that MilliQ water was used as the solvent. The spiking and calibration standard solutions were kept at 4°C. MAA, EAA, and BAA in isopropanol were found to be stable for at least six months, and the concentrations of the solutions prepared in MilliQ water did not change within two months under these storage conditions. 2-Bromopropionic acid (BrPA, 99%, Supelco, Bellefonte, PA) was the internal standard prepared in methyl-tert-butyl ether (MTBE) at 0.25 mg/mL. n-Hexane, dichloromethane, methanol, isopropanol, and MTBE (high-performance liquid chromatography or pesticide grade) were obtained from either EMD (Gibbstown, NJ) or BDH (Toronto, Canada). Diethyl ether, 99.9%, spectrophotometric grade without inhibitors, was obtained from Sigma-Aldrich Chemicals. Reagent-grade water was prepared with a MilliQ (Millipore, Bedford, MA) water purification system. N-Nitroso-N-methyl-p-toluenesulphonamide (Diazald) and diethylene glycol monoethyl ether (Carbitol) were purchased from Sigma-Aldrich. Silicic acid was obtained from J.T. Baker (Phillipsburg, NJ). (Trimethyl)silyl diazomethane (TMSDM), a solution of 2 mol/L in n-hexane, was procured from Fluka (Steinhein, Switzerland). Bond Elut* Na2SO4 cartridges (1.4 g) were purchased from Varian (Palo Alto, CA). Bakerbond® C18 cartridges, 500 mg/3 mL, were obtained from J.T. Baker.

**Sample treatment**

Urine samples from aliphatic alkyl ether-treated rats were collected from a study in this laboratory and kept at −80°C before use (2). Urine used as control (blank) was obtained from experimental rats that were not dosed with any ether. The aliphatic alkyl ethers studied were 1,6-dimethoxyhexane (DMHx), 1,1-dimethoxyheptane (DMHp), 1-ethoxyhexane (EHpE), 1-ethoxyheptane (EHxE), 1-isopropoxyoctane (IPO), 1-methoxyheptane (MHpE), 1-methoxyoctane (MOE), 1-propoxyhexane (PhxE), and dibutyl ether (BE). The molecular structures of the ethers are shown in Figure 1.

SPE was carried out with a Supelco 12-port Visiprep SPE Vacuum Manifold. Before sample application, C18 cartridges were conditioned with 3 mL of methanol and 5 mL of water sequentially. A 1-mL urine sample was acidified with 100 μL of concentrated HCl, applied to the cartridges with a Pasteur pipet, and allowed to flow through by gravity. After sample application, the cartridges were centrifuged at 3,000 rpm for 5 min, placed on the SPE vacuum manifolds, and air-dried for 15 min with an air-stream generated from a vacuum pump. Each of the C18 cartridges was then connected to a Na2SO4 cartridge. The serially connected cartridges were eluted with 2 mL of 2.5% isopropanol in diethyl ether. The residual water was removed as the eluate flowed through the Na2SO4 cartridge.

One hundred microliters of the BrPA internal standard solution was added to the eluate, and the solution was concentrated at ambient temperature to a final volume of 0.05 mL with a gentle stream of nitrogen. The concentrates were reconstituted with n-hexane to a volume of 0.2 mL. Derivatization with diazomethane was conducted using a modification of the United States Environmental Protection Agency method 515.1 (22). Diazald and 37% KOH water solution were allowed to react and generate diazomethane as the derivatizing reagent. Before the reaction, diazald was dissolved in a 50% diethyl ether and 50% carbitol solution at 100 mg/mL. The solution was stored in an amber bottle in a refrigerator and was stable for 1 month. The diazomethane generated was bubbled through 1/16" PTFE tubing into the n-hexane solution with the aid of a gentle stream of nitrogen at a rate of 10 mL/min until the sample turned yellowish. Despite the very small volumes of diazomethane used, great care was taken to avoid human exposure to this toxic substance. All the work was conducted in a well-ventilated fume hood, and long tweezers were used to place the outlet of PTFE tubing into the sample. The outlet of the PTFE tubing was rinsed with n-hexane between samples to avoid cross contamination. After 30 min, the diazomethane in the sample was destroyed by addition of silicic acid.

The SPE method was compared with liquid–liquid extraction (LLE) for extraction efficiency. For LLE, 1 mL of urine sample was acidified with 100 μL of concentrated HCl. The analytes were then extracted with 1 mL of 2:1 (v/v) of methylene chloride and isopropanol. One hundred microliters of internal standard was added, and the sample was derivatized with diazomethane.

In this work, extraction recoveries were used to monitor the extraction efficiency of the analytes from urine. Extraction recoveries were obtained by use of calibration standards in pure solvent rather than of matrix extract. Also, to obtain the extraction recoveries, the internal standard was added after the spiked analytes were extracted from the sample matrix.

For comparison, TMSDM was also studied as an alternate derivatizing agent. For derivatization with TMSDM, following the method of Sakai et al. (8), 10 μL of TMSDM solution

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Figure 1. Molecular structures of nine aliphatic alkyl ethers.
(2 mol/L in n-hexane), instead of diazomethane, was added to 0.2 mL of sample solution.

**Instrumental analysis**

GC–MS analysis was performed with a Thermo Finnigan Trace 2000 GC coupled to a PolarisQ MS. The instrument was equipped with an AS 2000 liquid autosampler, a Supelco OMEGA WAX 250 fused-silica capillary column (30 m × 0.25 mm × 0.25 μm), and Xcalibur data system. The oven temperature was programmed at the initial temperature of 50°C, held for 2 min, and then increased at a rate of 2°C/min to 120°C. The oven was held at this temperature for 1 min, then increased at 30°C/min to 220°C, and held for 3 min. Helium was the carrier gas with a flow rate of 0.8 mL/min, and the split flow was 32 mL/min. A 2-μL volume of sample solution was injected with a splitless injection mode and a purge on time of 1.0 min. The temperatures of the injection port, transfer line, and MS ion source were set at 220°C, 250°C, and 200°C, respectively. The selected ion monitor (SIM) was used for quantification. A typical GC–MS total ion chromatogram (TIC) for 1 mL of blank rat urine sample (A) and 1 mL of blank rat urine spiked with MAA, EAA, and BAA with a concentration of 20, 10, and 5 pg/mL, respectively, (B) are shown in Figure 2.

Some of the samples were also analyzed using a GC–FID in parallel to analysis of the GC–MS. A GC (Hewlett Packard model 5890) equipped with an autosampler (Agilent 6890 Serials) and Agilent Chemstation data system was used. The GC capillary column, chromatographic temperature program, and carrier gas flow rates were the same for GC–MS and GC–FID analyses. The temperature of FID was set at 260°C, and nitrogen (30 mL/min) was the makeup gas. The flow rate of hydrogen and air was 30 and 350 mL/min, respectively. Splitless injection was used. Other conditions were: sample injection volume, 2.0 μL; and purge on time, 0.5 min. FID is less selective than GC–MS–SIM. Typical chromatograms with FID for the previously described blank and spiked urine samples are shown in Figure 3.

**Results and Discussion**

**Efficiency of SPE for isolation of alkoxyacetic acids from urine samples**

A comparison of recoveries of MAA, EAA, and BAA using SPE and a LLE method from Shih et al. (19) is shown in Table I. As one can see, much higher extraction efficiencies have been achieved with SPE than with LLE for the alkoxyacetic acids, especially for MAA and EAA. The average recoveries for MAA and EAA were 87% and 97% with this SPE method, in contrast to 27% and 43% with the LLE.

The SPE recoveries of MAA, EAA, and BAA were little affected by the concentrations of the analytes spiked into a urine sample. This is illustrated with the good linearity of plots of the area ratio of the analytes to the internal standard versus the concentration of the analytes that were spiked into 1 mL of

![Figure 2. TIC of GC–MS–SIM of blank rat urine sample (A) and blank rat urine sample spiked with MAA (20 μg/mL), EAA (10 μg/mL), and BAA (5 μg/mL) (B). Internal standard (BrPA) concentration: 5 μg/mL.](image)

![Figure 3. Chromatogram of extract of blank rat urine sample (A) and blank rat urine sample spiked with MAA (20 μg/mL), EAA (10 μg/mL), and BAA (5 μg/mL) (B). Internal standard (BrPA) concentration: 5 μg/mL.](image)

**Table I. Comparison of Recoveries and RSDs of MAA, EAA, and BAA Obtained with SPE and LLE (19)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>SPE Recovery (%)</th>
<th>SPE RSD (%)</th>
<th>LLE Recovery (%)</th>
<th>LLE RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA</td>
<td>87</td>
<td>1.7</td>
<td>27</td>
<td>7.9</td>
</tr>
<tr>
<td>EAA</td>
<td>97</td>
<td>0.7</td>
<td>43</td>
<td>9.0</td>
</tr>
<tr>
<td>BAA</td>
<td>109</td>
<td>3.7</td>
<td>85</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*Sample: 1 mL of rat urine spiked with MAA, EAA, and BAA with a concentration of 20, 10, and 5 μg/mL, respectively. Recoveries and relative standard deviations (RSDs) are an average of 4 replicates.
urine over an 80-fold range of concentrations (Figure 4). The correlation coefficients ($R^2$) of the linear plot are generally over 0.999. C$_{18}$ cartridges from different suppliers may have a slight difference in properties, such as adsorbent particle size and distribution, pore size, specific area, carbon content, etc. C$_{18}$ cartridges from several suppliers were tested (data not shown). The Bakerbond C$_{18}$ is the one with which the best extraction efficiency of MAA, EAA, and BAA was obtained.

The alkoxyacetic acids are weak acids. The $pK_a$ of the acids were not directly available from the literature, but they could be speculated to be around 2 from the $pK_a$ of related compounds. In urine samples that normally have a pH of 5–8, they are ionized. To isolate the analytes from urine with non-polar C$_{18}$ bonded silica, the samples need to be acidified. Adding 100 μL of concentrated HCl to 1 mL of urine leads to a pH between 0.6 and 0.9. The alkoxyacetic acids should exist as neutral molecules at this pH. Isolation of the analytes from urine has been demonstrated to be difficult, even though the acids were converted to their neutral molecules. LLE has been widely used for isolation of the alkoxyacetic acids from urine samples (7–16,19). The LLE method was systematically optimized by Sakai et al. (8) and Shih et al. (9,19), and it was found that a mixture of 2:1 methylene chloride and isopropanol gave the best extraction efficiency. The comparison of our SPE with Shih et al.'s LLE shows that SPE is advantageous in that it gave a much higher extraction efficiency for the most hydrophilic alkoxyacetic acids, MAA and EAA. LLE was demonstrated to be

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Plot of peak area ratio of MAA, EAA, and BAA to internal standard versus their spiking concentrations.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Mass spectrum of MAA (A), EAA (B), and BAA (C) methyl ester.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Plot of concentration of MAA measured with GC-FID versus those measured with GC-MS.
a simple method for analysis of the alkoxyacetic acids in urine, and by choosing proper calibration, satisfactory results were obtained (8,19). However, low extraction recovery may limit the sensitivity of the overall method, and the analysis result tends to be more affected by the change of sample matrix. SPE, with a commercially available C18 cartridge, is also simple and, therefore, may provide an alternative analytical method for the hydrophilic alkoxyacetic acids in urine. Another advantage of SPE is that the analytes may be eluted from the cartridge with a broad range of organic solvents or their mixtures. This makes sequential concentration and derivatization of the acids easy.

Optimization of GC–MS method

The mass spectra of methyl esters of MAA, EAA, or BAA usually show weak molecular ions, but show a characteristic fragment ion of m/z 74, which is an ion from McLafferty rearrangement of acetate methyl ester group—CH3COOCH3. There is some difference of the mass spectra between this work and those reported by Sakai et al. (8) in that the relative peak heights of m/z 74 to the base peaks (m/z 45, 31, and 41 for MAA, EAA, and BAA, respectively) is much lower. This may be because of different MSs and tuning method being used. Quantification of MAA, EAA, and BAA was carried out in SIM mode by choosing m/z 74 rather than the base peaks because the former can give a more stable chromatogram baseline and higher sensitivity. Reducing the electronic energy of the MS ion source from 70 to 60 eV was found to be effective to improve the sensitivity of SIM quantification of the acids. The profiles of mass spectra of methyl esters of MAA, EAA, and BAA at 60 eV are almost the same as those at 70 eV, but the ratio of m/z 74 to base peak is increased by more than 100%. Consequently, the SIM detection limit at 60 eV is one half to a third of that at 70 eV. A further lowering of the electronic energy, from 60 to 50 eV, offered no improvement in the sensitivity. At 60 eV, the lowest detection limits for MAA, EAA, and BAA were 0.04, 0.02, and 0.02 ng, respectively, with a signal-to-noise ratio (S/N) of 3. When 1 mL of urine sample was used and the sample solution volume prior to GC–MS analysis was 0.2 mL, the lowest detection concentrations for MAA, EAA, and BAA were estimated to be 4, 2, and 2 ng/mL (ppb), respectively.

Comparison of GC–MS and GC–FID for quantification

Some of the rat urine samples were analyzed with both GC–FID and GC–MS. A plot of concentrations of MAA measured with GC–MS versus those measured with GC–FID for 47 samples is presented in Figure 6. For analysis of the acids, 0.2 mL of urine sample was used. It can be seen that the concentrations measured with the two methods agree well. Some of the large relative differences occur for samples with low MAA content. This may be due to the low selectivity of FID. With FID, interference to the determination of acid analytes could be found because of their low resolution with some of the components that were coextracted from urine matrix. The interference from coextracted components is significant for samples where the analyte concentrations are close to the detection limit of the FID.

The detection limits of MAA, EAA, and BAA with GC–FID were estimated to be 0.4, 0.2, and 0.1 ng for S/N = 3, which is 5 to 10 times higher than those for GC–MS. The practical lowest detection concentrations with GC–FID are higher than those directly estimated from the previously described detection limits because of the low resolution of the analytes with the coextracted components from the urine samples.

Comparison of methods of derivatization

For GC analysis of the alkoxyacetic acids, the analytes are usually converted to their methyl (8–11,20), pentafluorobenzyl (PFB) (7–17), or butyldimethylsilyl ester (18). MS and FID are widely used for detection when the analytes were converted into their methyl esters. In the literature (7,15–17), the acids were also measured with an electron capture detector (ECD) after they were converted to their PFB ester. ECD is sensitive towards halogen-containing molecules. However, derivatization with PFB may generate numerous interfering products that make the GC separation and detection difficult, and sometimes, a further cleanup is needed (16,20). Methyl esters of the acids could be prepared with several alternative derivative approaches (8–11,20). The methyl ester prepared with diazomethane showed few interfering components that were brought into the sample through the derivatization process. (Trimethyl)silyl diazomethane is a simple methylation method for the acids, but it was found in this work that a lot of impurities were brought about from derivatization with the reagent. This makes it difficult for quantification, especially with the less selective FID detector.

Applications as biomarker of exposure/effects

A number of rat urine samples were analyzed. These samples were from a separate toxicological study of aliphatic alkyl ethers in this laboratory (23). MAA was determined in urine samples of rats dosed with 200 mg/kg/day DMHx at levels of 243.13 ± 110.05 versus 1.60 μg/mL in urine from control animals. In the same study, EAA was determined in the urine of rats dosed with EHxE, and BAA was determined in urine of rats dosed with butyl ether (BE), as shown in Table II. The identification of

### Table II. Levels of MAA, EAA, and BAA in Urine Collected from Male Rats Treated Orally with BE, EHxE, MHpE, and DMHx at a Dose Concentration of 200 mg/kg/day

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAA (μg/mL, Mean ± SD, n = 7)</th>
<th>EAA (μg/mL, Mean ± SD, n = 7)</th>
<th>BAA (μg/mL, Mean ± SD, n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.60 ± 0.53</td>
<td>0.16 ± 0.11</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>BE</td>
<td>1.74 ± 0.57</td>
<td>0.37 ± 0.05</td>
<td>243.65 ± 122.56*</td>
</tr>
<tr>
<td>EHxE</td>
<td>1.22 ± 0.54</td>
<td>104.17 ± 41.21*</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>MHpE</td>
<td>4.28 ± 1.27</td>
<td>0.10 ± 0.04</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>DMHx</td>
<td>242.13 ± 110.05*</td>
<td>0.11 ± 0.06</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Indicates significant difference (p < 0.05) from control based on Dunnett's test following parametric ANOVA.
the alkoxyacetic acids is an indication of the metabolic pathway of these alkyl ethers and can be used as a biomarkers of exposure. The concentrations of the acids also correlated with the dose levels of the alkyl ethers (data not shown). The quantities of various alkoxyacetic acids generated seemed to correlate to the levels of toxicity of various alkyl ethers. For example, DMH was found to be more toxic than other ethers in rats in previous work (2), which correlated well with high levels of MAA found in the urine of rats. The fact that BE-treated rats exhibited no testis toxicity, despite considerable conversion of this parent compound to BAA, agrees with published reports of the minimal testis toxicity of BAA (24).

Conclusions

A new method based on SPE and GC was developed for analysis of urinary alkoxyacetic acids. SPE with a commercially available C18 bonded silica cartridge is a simple approach for isolation of the alkoxyacetic acids, and higher recoveries may be obtained with SPE than the traditional LLE. The conditions for GC-MS-SIM analysis of the alkoxyacetic acids were evaluated. An electron energy of 60 eV for the ion source gave optimal performance of the GC-MS in this work. The sensitivity with GC-MS could be 5 to 10 times higher than those with GC-FID and is the method of choice for determination of a low urinary concentration of MAA, EAA, and BAA.

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