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Short communication

Procedure for the quantification of the biomarker (2-methoxyethoxy)acetic acid in human urine samples

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Abstract

An accurate and precise procedure was developed for the detection and quantification of (2-methoxyethoxy)acetic acid (MEAA), a metabolite and biomarker for human exposure to 2-(2-methoxyethoxy)ethanol. The compound 2-(2-methoxyethoxy)ethanol has a wide array of industrial applications including its use as an additive in military jet fuel. Exposure to 2-(2-methoxyethoxy)ethanol is a health concern owing to its toxicity which includes developmental and teratogenic properties. Sample preparation consisted of liquid–liquid extraction (LLE) and esterification of MEAA to produce the ethyl ester. Measurement was by a gas chromatograph (GC) equipped with a mass selective detector (MSD) using a HP-1 capillary column. Recovery studies of spiked blank urine demonstrated good accuracy and precision; recovery varied between 95 and 103% with relative standard deviations of 8.6% and less. The limit of detection (LOD) for this procedure was found to range from 0.02 to 0.08 μg/ml equivalent levels of MEAA in urine. These data and other aspects of the validation of this procedure will be discussed.
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1. Introduction

The toxicity of glycol ethers and related compounds was known as early as 1939 [1] and has been reported in the literature [2–5]. The compound 2-(2-methoxyethoxy)ethanol has many industrial applications including the use as an anti-icing agent in JP-8 military jet fuel. It is a health concern because of its similarity to 2-methoxyethanol, a glycol ether with significant prenatal toxicity [4,6]. The toxicology data for 2-(2-methoxyethoxy)ethanol has been reviewed [2], and the compound has been studied numerous times. Hardin et al. [7] found that 2-(methoxyethoxy)ethanol had teratogenic and developmental toxicity in Sprague–Dawley rats, and Yamano et al. [8] demonstrated teratogenic properties in Wistar rats. Scortichini et al. [9] observed fetotoxicity in the rabbit from dermal exposure. Dermal exposure to 2-(2-methoxyethoxy)ethanol during JP-8 fuel-transfer and aircraft tank-cleaning is a possible exposure route for air force personnel and represents an occupational health study in progress by this laboratory. (2-Methoxyethoxy)acetic acid

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(MEAA) has been shown to be the urinary metabolite best suited for use as a short-term biological marker of exposure to 2-(2-methoxyethoxy)ethanol [10]. Fig. 1 shows the metabolic pathway for 2-(2-methoxyethoxy)ethanol to MEAA. However, as is shown in Fig. 1, other metabolites can be formed from the initial alcohol by glucuronyl transferase, dealkylase or sulfotransferase. MEAA can be further metabolized by acyltransferase or dealkylase carboligase. The focus of this manuscript is the development and validation of a method for the detection and quantification of MEAA.

It was the objective of this study to develop a simple and effective procedure to detect and measure the levels of MEAA in human urine samples. It was also a goal to have a validated [11,12] test method in place for use in monitoring exposed individuals in the United States Air Force and to have the methodology in place to monitor animal exposure during toxicity experiments, both of which are part of studies in progress or proposed at this laboratory. The analysis procedure was designed to be simple and straightforward. Liquid–liquid extraction (LLE) has been noted in the literature for its inherent simplicity and ease of use over other sample extraction and enrichment techniques [13] and was ultimately chosen for the extraction of MEAA from urine in this study. Esterification of MEAA to the corresponding ethyl acetate proved to be effective; a similar esterification procedure has been reported by Laitinen [14] for the analysis of butoxyacetic acid using the methyl ester formation. Esterification of organic acids with various alcohols for gas chromatographic (GC) analysis is frequently practised; haloacetic acids [15], mandelic acid [16,17] and 3-phenoxybenzoic acid [18] are just a few examples of urine extract analysis using acid catalyzed esterification reported in the literature. Other GC alkoxyacetic acid urine analysis methods reported have followed extraction with derivatization using diazomethane [19], pentafluorobenzyl bromide [20–23] or trimethylsilyldiazomethane [24]. These methods are generally more complicated than esterification. Diazomethane is hazardous for the laboratory chemist and pentafluorobenzyl bromide derivatization can generate irritating hydrogen bromide gas. Trimethylsilyldiazomethane can be expensive to use. Acid catalyzed esterification avoided many of these difficulties. Capillary GC using a HP-1 column and detection by means of a mass selective detector (MSD) proved to be selective for the method and eliminated interferences for the analysis of the urine sample matrix. Deuterated 2-butoxyacetic acid (d-BAA) was chosen as an internal standard for the chromatographic procedure.

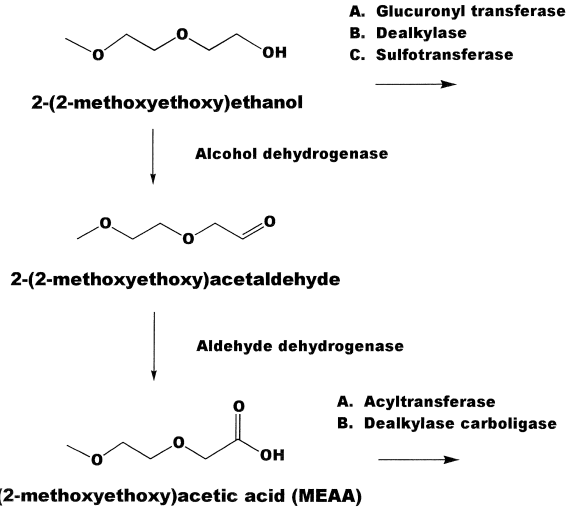

Fig. 1. Metabolic pathways for 2-(2-methoxyethoxy)ethanol. (2-Methoxyethoxy)acetic acid, MEAA, is a major metabolite and biomarker of exposure and is the focus of this analysis procedure. 2-(Methoxyethoxy)ethanol can also be metabolized by glucuronyl transferase, dealkylase or sulfotransferase. MEAA can be metabolized further by acyltransferase or dealkylase carboligase.

2. Experimental

2.1. Instrumentation and chromatographic conditions

The chromatographic analysis was carried out using an Agilent Technologies model 6890 gas chromatograph (Avondale, Pennsylvania, 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 240 °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 50 °C and held for 1 min, then increased to 140 °C at a rate of 3 °C/min and finally increased to 230 °C at a rate of 15 °C/min. A post run of 240 °C for 5 min was included with each run. 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 59 (MEAA) and ion m/z 66 (d-BAA) 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. The injection size of the final solution was 0.5 μl using splitless mode injection.

2.2. Chemicals and reagents

Standard compounds of (2-methoxyethoxy)acetic acid (MEAA, CAS no. 16024-56-9) and deuterated 2-butoxyacetic acid (d-BAA) were synthesized and described previously by Cheever et al. [25] and by Brown et al. [26]. All other reagents used were of analytical grade and are regularly available in a laboratory.

2.3. General urine sample preparation

Actual urine samples or MEAA-spiked blank urine samples were treated identically. A 4.0-ml portion of the urine was placed in a screw-capped tube and acidified with 20 μl of concentrated (12 *M*) hydrochloric acid. A 0.5-ml aliquot of a 40 μg/ml d-BAA internal standard solution was added. A 0.5-ml portion of deionized water for test samples or standard MEAA 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 by 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. A 3.0-ml portion of methylene chloride was added to each esterified urine 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 were combined and dried with anhydrous magnesium sulfate. The methylene chloride solutions were reduced to 1 ml volume by evaporation by nitrogen sweep at room temperature. These concentrated solutions were placed in crimp-capped vials for GC analysis.

2.4. Standard sample preparation and recovery studies

MEAA standards for calibration were prepared at the 0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40 and 50 μg/ml equivalent levels in urine plus a blank 0 μg/ml level sample. Blank urine was spiked at the 2, 10, and 20 μg/ml equivalent MEAA level for each experimental day of the primary recovery study. A secondary recovery study consisted of collecting urine from 20 non-exposed volunteers. Urine samples containing no MEAA or d-BAA internal standard, urine samples with d-BAA only, and urine samples spiked with 10 μg/ml equivalent MEAA level and d-BAA internal standard were prepared for this second recovery study.

2.5. Calculations

Calculations were based on peak area ratios of MEAA to d-BAA. Standard calibration curves were linear within the 0.1 to 50 μg/ml MEAA range used; correlation coefficients were 0.98 or greater and *y*-intercepts approached zero for all curves generated with this method. Two calibration curves, at the beginning and end of the run, using all the standards were collected for each batch set of the first recovery study. This generated six curves for data presented for the first recovery study. Three calibration curves, at the beginning, middle, and end of a run, were collected during the second recovery study. Since two batch runs were made during the second recovery study of spiked unexposed urine, this created an additional six calibration curves. Calibration curve slope drift was minimal; less than 2% was

observed within any batch run. Duplicate injections were performed for all spiked samples and average values calculated for the recovery data.

The limit of detection (LOD) was calculated in the traditional way, three times noise level divided by the slope of the calibration curve [11]. The noise level was determined for each batch run by integrating noise levels in chromatograms at the retention time window for MEAA from the 0 μg/ml blank standard. At least five chromatograms were used, and the average noise level based on height was used. The slope from the calibration curve using peak height ratios of the standard levels was used for this calculation.

3. Results and discussion

3.1. Chromatographic separation and specificity

The optimized chromatographic conditions developed for this procedure proved to be selective and have no major interferences. The unspiked urine samples chromatographed showed no interfering peaks for MEAA; the blank samples from 20 non-exposed volunteers showed chromatograms with no interferences for either MEAA or d-BAA internal standard. Therefore, this procedure appears to be specific for MEAA. Typical total ion chromatograms from urine of a non-exposed volunteer are shown in Fig. 2. The first chromatogram (Fig. 2A) shows a blank chromatogram from a non-spiked urine sample, the ethyl esters of MEAA and d-BAA are shown in the second chromatogram (Fig. 2B). The baseline displayed no major drift and the ethyl ester derivative peaks had over a 1-min separation in retention times. Peak shape was excellent and there was no evidence of any carry over between injections; blank urine samples injected after the 50 μg/ml standard displayed no MEAA ethyl ester peak. Shih et al. [27] reported the analysis of alkoxyacetic acids directly by gas chromatography without a derivatization step, however, MEAA showed extensive injection carryover and peak ghosting on many chromatographic columns and conditions tried early during this study. Esterification of MEAA eliminated these problems.

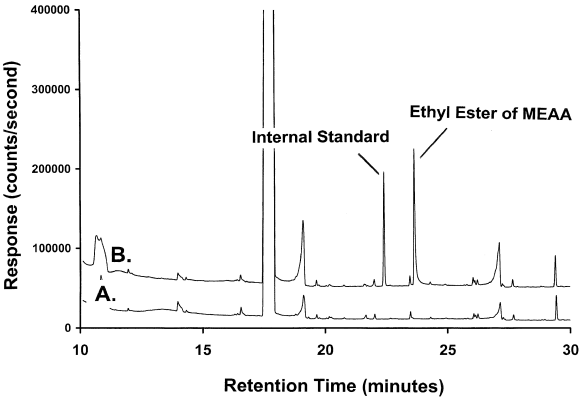

Fig. 2. (A) A total ion chromatogram of a blank non-exposed volunteer urine sample analyzed by the described procedure. (B) A total ion chromatogram of a 10 μg/ml spiked MEAA urine solution with 5 μg/ml equivalent d-BAA used as the internal standard. No interfering peaks were evident in any of the group of 20 non-exposed volunteer samples.

The mass selective detector was useful in adding additional selectivity to the procedure. The ion m/z 59 [$CH_3OCH_2CH_2-$] was chosen for monitoring for the calibration curve used in the calculations because of its greater abundance, and it was a characteristic fragment for the MEAA ethyl ester. Ion m/z 66 [$CD_3CD_2CD_2CD_2-$] was monitored for the ethyl ester of d-BAA, the internal standard, for the same reasons. The use of ion m/z 58 [$-OCH_2C{=}O$] for a qualifying ion was considered initially for the MEAA ethyl ester, however, no chromatographic interferences or co-eluting analytes were observed in any part of the development of this method for either the analyte or the internal standard. Therefore, qualify ions were not considered necessary.

3.2. Liquid–liquid extraction and the selection of the internal standard

The liquid–liquid extraction (LLE) proved to be useful for this procedure. During early development work, a solid-phase extraction procedure designed for these acidic compounds using Waters Oasis® MAX cartridges (anion-exchange and reversed-phase mixed mode SPE cartridge) was attempted. However, linearity for the extraction of MEAA was poor

at higher levels and recovery of the compound was problematic. Extensive procedural development time to determine bed capacity optimization and the linear extraction range was not desired, so this procedure was not pursued. Also, the validation of the final procedure using LLE gave excellent results.

Deuterated 2-butoxyacetic acid, d-BAA, was chosen as an internal standard, because this compound would not be expected to be present in a worker exposed to glycol ethers. Generally, an internal standard compensates for changes in solvent volume, however, the use of d-BAA as a procedural internal standard reduces analysis variation to acceptable levels. Variability caused by differences in the extraction and derivatization procedure can be compensated for by the use of d-BAA added to the initial urine sample. d-BAA is chemically similar to MEAA and has similar solubility and extraction properties. Good calibration curves were obtained using this internal standard and the precision of the recovery data implies a reproducible extraction and esterification of both MEAA and d-BAA. Additionally, the use of an internal standard increases the precision of chromatographic injections. Five replicate injections of the 10 μg/ml standard sample gave relative standard deviations (RSD) of peak area ratios ranging from 0.1 to 1.9% during the recovery studies.

3.3. Analyte recovery

A primary recovery study of blank urine spiked with MEAA was performed over three separate experimental batch runs to demonstrate the accuracy and precision of the procedure. These data are presented in Table 1A; average recovery was between 95 and 103% for the three MEAA spiked sample levels investigated. For each run, the experimental trial consisted of three samples at three concentration levels. The 2 μg/ml level had the lowest average recovery at 95%, and the 20 μg/ml had an average recovery of 103%. This is within statistical expectations for recovery and displays no obvious bias. The highest relative standard deviation was 7.8% for the 10 μg/ml, which was considered acceptable for a procedure of this nature. Most of the variation on recovery would be attributed to the two extraction steps and the esterification step within the sample preparation of this procedure. The second recovery study used urine samples from 20 non-exposed volunteers and demonstrated that the procedure was both accurate and precise (Table 1B). The 10 μg/ml spiked samples show an average recovery of 103% and a relative standard deviation (RSD) of 8.6%. The lowest individual sample recovery was 8.9 μg/ml and the highest was 12.0

Table 1
Recovery studies of (2-methoxyethoxy)acetic acid; (A) multilevel recovery study of MEAA from spiked urine samples[a], (B) recovery of 10 μg/ml MEAA spikes from urine samples of 20 non-exposed volunteers[b]

(A) Spike level (μg/ml)	Mean MEAA recovered ($n=9$) (μg/ml)	Average % recovery	SD (μg/ml)	% RSD
2	1.89	95	0.11	5.8
10	10.0	100	0.78	7.8
20	20.6	103	1.33	6.4

(B) Mean recovery (μg/ml)	Average % recovery	Lowest value (μg/ml)	Highest value (μg/ml)	% RSD
10.3	103	8.9	12.0	8.6

[a] 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 column was used on trial run 3.

[b] All non-spiked samples showed no MEAA ethyl ester peak in the chromatograms. The second recovery study was performed using a third HP-1 column, one different from those used in the first recovery study above.

μg/ml. The second recovery study also would indicate that differences in individual urine samples do not cause matrix variation which would affect recovery significantly. Again, no interferences were noticed in any of the samples analyzed.

3.4. Method reproducibility, limit of detection and analyte stability

Three different HP-1 capillary columns of different manufacturing lots were used during these recovery studies; the results are, therefore, expected to be consistent and reproducible with different HP-1 GC columns. This would indicate robustness for the chromatography used in this procedure. The limit of detection (LOD) was found to range from 0.02 to 0.08 μg/ml MEAA equivalent levels in urine. General condition of the columns used and day-to-day variation in detector noise accounted for this range. Aqueous stock standard solutions of MEAA stored for 3 weeks gave full recovery assay values when compared to a freshly prepared MEAA standard; stability is implied for this period of analysis time. This procedure should be considered accurate for estimation of MEAA in human urine within the 0.1 to 50 μg/ml standard range. Field samples at higher levels can be diluted to a concentration within that range for analysis.

3.5. Future work

An accurate and precise method to monitor the level of MEAA in human urine has been developed and validated. Planned future work includes the analysis of actual urine field samples collected from air force personnel. This field sample work is part of a larger comprehensive study which is beyond the scope of this short communication, and it will be reported in detail elsewhere. Modification of this procedure for use in monitoring urine collected from exposed rodents in toxicity studies is also in progress.

References

[1] E.P. Laug, H.O. Calvery, H.J. Morris, G. Woodard, J. Ind. Hygiene Toxicol. 21 (1939) 173.
[2] V.K. Rowe, M.A. Wolf, Derivatives of glycols, in: G.D. Clayton, F.E. Clayton (Eds.), 3rd rev, Patty's Industrial Hygiene and Toxicology, Vol. 2C, John Wiley, New York, 1982, p. 3909.
[3] Occupational Exposure to Ethylene Glycol Monobutyl Ether and Ethylene Monobutyl Ether Acetate, US Department of Health and Human Services, 1990, NIOSH Publication no 90-118.
[4] Occupational Exposure to Ethylene Glycol Monomethyl Ether, Ethylene Glycol Monoethyl Ether and Their Acetates, US Department of Health and Human Services, 1991, NIOSH Publication no. 91-119.
[5] D.W. Hobson, A.P. D'Addario, R.H. Bruner, D.E. Uddin, Fundam. Appl. Toxicol. 6 (1986) 339.
[6] K.L. Cheever, T.F. Swearengin, R.M. Edwards, B.K. Nelson, D.W. Werren, D.L. Conover, D.G. DeBord, Toxicol. Lett. 122 (2001) 53.
[7] B.D. Hardin, P.T. Goad, J.R. Burg, Fundam. Appl. Toxicol. 6 (1986) 430.
[8] T. Yamano, T. Noda, M. Shimizu, S. Morita, M. Nagahama, Arch. Environ. Contam. Toxicol. 24 (1993) 228.
[9] B.H. Scortichini, J.A. John-Green, J.F. Quast, K.S. Rao, Fundam. Appl. Toxicol. 7 (1986) 68.
[10] D.E. Richards, K.B. Begley, D.G. DeBord, K.L. Cheever, W.W. Weigel, M.A. Tirmenstein, R.E. Savage, Arch. Toxicol. 67 (1993) 531.
[11] J.M. Green, Anal. Chem. 68 (1996) 305A.
[12] The Federal Register 60 (1995) 11260.
[13] A.C. Hogenboom, W.M.A. Niessen, U.A.Th. Brinkman, J. Sep. Sci. 24 (2001) 331.
[14] J. Laitinen, Sci. Total Environ. 199 (1997) 31.
[15] F. Wu, W. Gabryelski, K. Froese, Analyst 127 (2002) 1318.
[16] M. Korn, R. Wodarz, W. Schoknecht, H. Weichardt, E. Bayer, Arch. Toxicol. 55 (1984) 59.
[17] S. Kezic, I. Jakasa, M. Wenker, J. Chromatogr. B 738 (2000) 39.
[18] J. Hardt, Fresenius J. Anal. Chem. 371 (2001) 787.
[19] D. Groseneken, E. Van Vlem, H. Veulemans, R. Masschelein, Br. J. Ind. Med. 43 (1986) 62.
[20] D. Groeseneken, H. Veulemans, R. Massschelein, E. Van Vlem, Int. Arch. Occup. Environ. Health 61 (1989) 243.
[21] G. Johanson, H. Kronborg, P.H. Naslund, M.B. Nordqvist, Scand. J. Work Environ. Health 12 (1986) 594.
[22] G. Johanson, Arch. Toxicol. 63 (1989) 107.
[23] A.W. Smallwood, K.E. DeBord, L.K. Lowry, Environ. Health Perspect. 57 (1984) 249.
[24] T. Sakai, T. Araki, Y. Mauyama, Occup. Environ. Health 64 (1993) 495.
[25] K.L. Cheever, D.L. Richards, W.W. Weigel, J.B. Lal, A.M. Dinsmore, F.B. Daniel, Toxicol. Appl. Pharmacol. 94 (1988) 150.
[26] K.K. Brown, K.L. Cheever, M.A. Butler, P. Shaw, J.L. McLaurin, J. Chromatogr. B (2003) in press.
[27] T.-S. Shih, J.-S. Chou, C.-Y. Chen, T. Smith, Occup. Environ. Med. 56 (1999) 460.