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# A Test Procedure for the Determination of (2-Methoxyethoxy)acetic Acid in Urine from Jet Fuel-Exposed Mice

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**A test procedure for the determination of (2-methoxyethoxy)acetic acid (MEAA) was adapted and applied to urine samples from jet fuel (JP-8)-exposed mice using capillary gas chromatography with a mass selective detector (MSD). MEAA is a metabolite and proposed biomarker for exposure to 2-(2-methoxyethoxy)ethanol, a glycol ether component in the formulation of JP-8. The collected urine samples were spiked with deuterated butoxyacetic acid internal standard, and extracted with ethyl acetate, and esterified with ethanol and sulfuric acid, and the esters of the glycol ethers were extracted with methylene chloride. The chromatographic conditions used easily separate the MEAA ethyl ester from interferences within mouse urine. The application of this procedure to urine samples collected from mice demonstrated that MEAA was detectable after oral (2000 mg/kg) or dermal (50 $\mu$L) exposure for 7 days to JP-8 at levels as high as 8.5 or 6.5 $\mu$g/mL, respectively. This pilot demonstration indicated that total urinary MEAA was a viable biomarker for the two routes of JP-8 exposure in laboratory mice.**

**Keywords** GC-MS, Glycol Ethers, Jet Fuel, JP-8, (2-Methoxyethoxy)acetic Acid, MEAA

## INTRODUCTION

The compound 2-(2-methoxyethoxy)ethanol [CAS 111-77-3, diethyleneglycol monomethyl ether] has many industrial uses. One of the applications of 2-(2-methoxyethoxy)ethanol of particular concern to this laboratory is its use in the formulation of JP-8, a military jet fuel. 2-(2-Methoxyethoxy)ethanol is used as an anti-icing agent in JP-8 at a concentration of 0.1% (v/v) (National Research Council 2003). 2-(2-Methoxyethoxy)ethanol is a glycol ether, a class of compounds having known toxicity for many decades (Laung et al. 1939) and often reported in the literature (Rowe and Wolf 1982; Hobson et al. 1986). 2-(2-Methoxyethoxy)ethanol has been found to have teratogenic and developmental toxicities in Sprague-Dawley rats (Hardin et al. 1986), and this compound displayed teratogenic properties in Wistar rats (Yamano et al. 1993). Observed fetotoxicity in the rabbit from dermal exposure to 2-(2-methoxyethoxy)ethanol has also been reported (Scortichini et al. 1986). This compound's chemical similarity to 2-methoxyethanol, another glycol ether with reported significant prenatal toxicity (Cheever et al. 2001), makes exposure to 2-(2-methoxyethyoxy)ethanol a health concern.

(2-Methoxyethoxy)acetic acid (MEAA) has been shown to be the urinary metabolite best suited for use as a short-term biomarker for exposure to 2-(2-methoxyethoxy)ethanol

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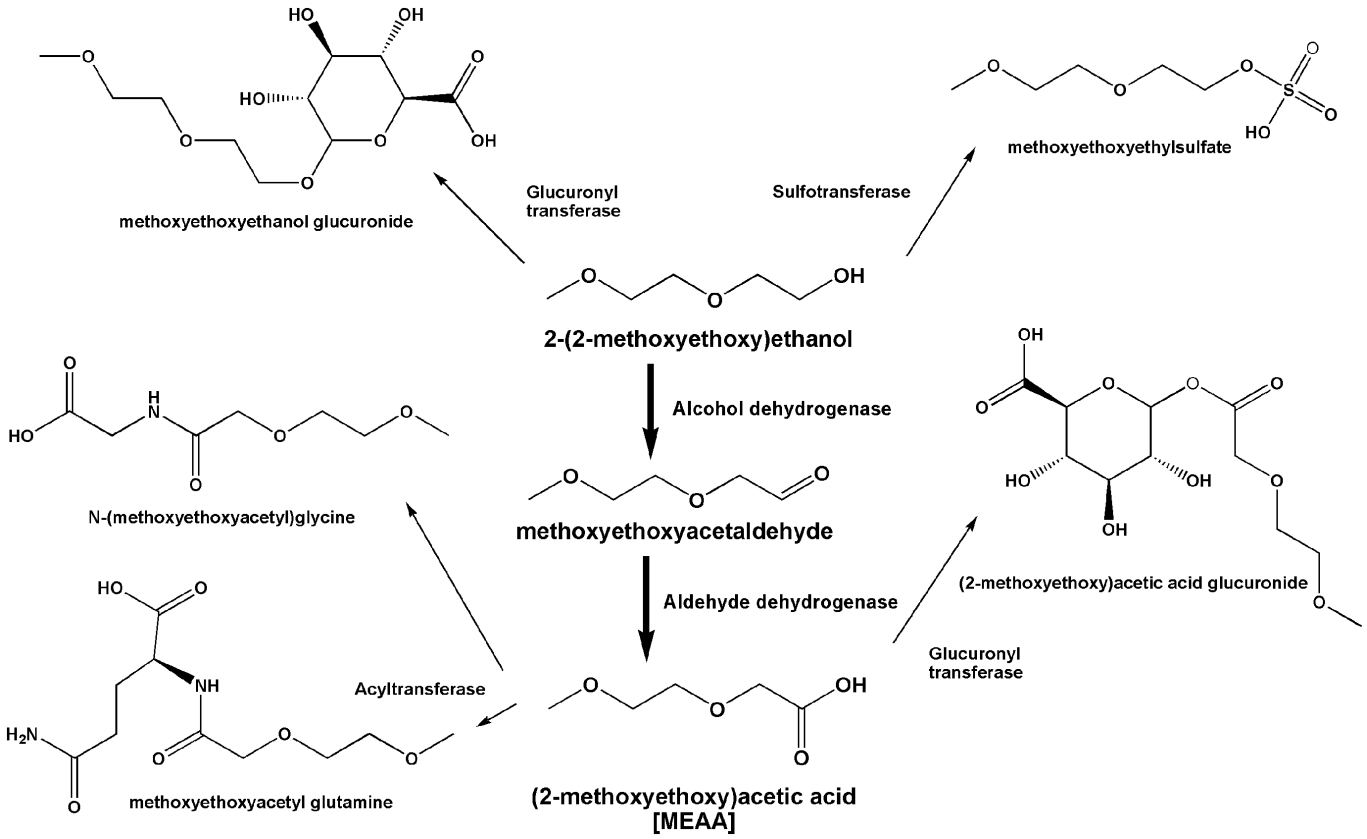


**FIG. 1.** Proposed metabolic pathway of 2-(2-methoxyethoxy)ethanol. (2-Methoxyethoxy)acetic acid, MEAA, is a major metabolite and the proposed biomarker of exposure for JP-8.

(Richards et al. 1993). The metabolism of 2-(2-methoxyethoxy)-ethanol is complex and is illustrated in Figure 1. In general, 2-alkylethanol compounds are rapidly metabolized via alcohol dehydrogenase to the corresponding alkoxyacetic acids and can be further metabolized by various mechanisms to glycine, sulfate, glutamate, and glucuronide conjugates of alkoxyacetic acids (Sumner et al. 1992). The conversion of 2-(2-methoxyethoxy)ethanol to MEAA by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (Cheever et al. 1988) is the pathway of interest for the preliminary study reported here. Other metabolites can be formed from the parent glycol ether by glucuronyl transferase, dealkyase, or sulfotransferase. MEAA can be further metabolized by acyltransferase or dealkylase carboligase. The focus of this manuscript is the adaptation and application of a test method for the quantification of MEAA in an evaluation of urine collected from exposed mice. This pilot study was desired because earlier work at this laboratory indicated that a profile of immunological dysfunction corresponds to JP-8 exposure in laboratory mice. The establishment of urinary MEAA as a quantifiable metabolite usable as a biomarker in a laboratory species exposed to JP-8 was an important goal of this work.

In the context of the presented known metabolism, the quantification of MEAA in urine from various animal species is necessary. Much is still not understood, and future toxicokinetic and pharmacokeinetic studies would be desirable for different animal species. The detection and quantification of MEAA from human urine using a validated test method has been previously reported (B'Hymer et al. 2003), but no test procedure has been applied to exposed animals in a controlled toxicological study. There were concerns at the initial stage of this study about the differences between the urine of humans and mice, which might complicate chromatographic separations or introduce interferences. The physical properties and chemical composition of mouse and human urine is variable, and differences noted for chromatographic composition may be explained by species differences in metabolism, diet, and activity cycle. Amounts of endogenous metabolites present depend on body weight and factors such as kidney function. Frequently the use of test procedures for one sample matrix, such as human urine, is not transferable to all animal species. Moreover, no test procedure has been presented for the quantification of MEAA in urine of animals that had been treated with JP-8 jet fuel, which is a complex mixture of chemicals. Various pilot toxicological studies using oral and dermal exposure routes in B6C3F1 mice are currently

ongoing or planned within this laboratory; therefore, validated test methodology for use in support of immunotoxicological studies was desired and the objective of this reported work.

The use of the mass selective detector (MSD) greatly increases the specificity of any test procedure for quantification. Specific ion masses characteristic of the analyte and internal standard are monitored and used for detection and quantification. Specificity of a test method or procedure is a key element of an analytical method validation (Green 1996) and was necessary for the application described here. Deuterated 2-butoxyacetic acid (d-BAA) was used as an internal standard for this procedure because it would not be found in test animals, and its characteristic ion mass would be specific using the mass selective detector. Also, it is chemically similar to the MEAA target analyte, and thus, it should extract and derivatize similarly. The sample preparation scheme and the analysis procedure were 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 such as solid-phase extraction (Hogenboom et al. 2001). Esterification of MEAA to the corresponding ethyl ester was also straightforward; acid-catalyzed esterification of organic acids with alcohols is commonly practiced in the literature (Hardt 2001; Laitinen 1997; Kezic et al. 2000; Korn et al. 1984; Wu et al. 2002) and was used for this assay procedure.

## MATERIALS AND METHODS

### Reagents and Chemicals

All reagents were of analytical grade. Standard reference compounds of (2-methoxyethoxy)acetic acid (MEAA, CAS no. 16024-56-9) and deuterated 2-butoxyacetic acid were synthesized and previously described (Cheever et al. 1988; Brown et al. 2003). JP-8 jet fuel was obtained from the United States Air Force, AFRL/HEST, Operational Toxicology Branch, Wright Patterson Air Force Base, Ohio. All other reagents were of analytical grade and are regularly available in a laboratory.

### Animals, Dosing, and Urine Collection

*Animal Care*

B6C3F1 female mice (8- to 9-week-old mice; Taconic, Germantown, New York) were maintained in an AAALAC-accredited facility during the study. Four to five animals were housed per cage and administered food (Harlan-Teklab, Madison, Wisconsin) and water ad libitum. Mice acclimated to the conditions of the treatment room (12-h light/dark cycle, 22 ± 2°C, 60 to 65% relative humidity) for 1 week prior to commencing the study. Bedding, food, and water were changed twice a week and the mice were observed daily.

*Animal Dosing*

JP-8 was administered either by oral gavage or dermal exposure. When administered orally, the mice were group-housed and JP-8 was delivered in a vehicle of olive oil with volumes of 80 to 120 $\mu$L that were adjusted to the body weight for each individual mouse to achieve accurate exposures of 500, 1000, or 2000 mg/kg. Vehicle-control animals were gavaged with 100 $\mu$L of olive oil. For the dermal exposures, mice were single-housed in ventilated cages to limit oral ingestion of JP-8 that might be attributed to grooming of cagemates. An area approximating 2 $cm^2$ on the dorsal thorax area of the mice was shaved one day prior to the application of JP-8. Neat JP-8 was delivered to the shaved site in amounts of 10, 50, or 75 $\mu$L per mouse. Dermal control animals were housed similarly and received 50 $\mu$L (Table 1) or 75 $\mu$L (Table 2) dermal applications of either acetone or olive oil as the negative controls. (Acetone has the property of evaporating quickly from the skin and was thought not to cause any adverse effects to the animals.) All exposures were administered daily on consecutive days during the 7-day dosing study (Table 1). During the time-course study (Table 2), a single dose of JP-8 was administered. Orally exposed animals were immediately placed in metabolic cages following exposure. To lessen the initial inhalation exposure associated with the dermal application of JP-8 and ingestion of JP-8 due to grooming of cagemates, dermally exposed animals were placed individually in ventilated cages for approximately 15 min prior to group housing in the metabolic cages.

**TABLE 1**
Oral and dermal dose study (urine sample collected after 7 days of dosing)

| | Oral | | Dermal | | |
|---|---|---|---|---|---|
| Treatment | Oil | JP-8 | Oil | Acetone | JP-8 |
| Concentration of MEAA ($\mu$g/mL) | nd | 8.5* | nd | nd | 6.5* |

Notes:
Assay values are $\mu$g MEAA per mL urine.
Oral = 2000 mg/kg dose of JP-8 in a vehicle of olive oil.
Dermal = all exposures were 50 $\mu$L dose volumes.
nd = none detected.
*Value is the average of two sample determinations on two sets of combined urine.
All values represents the collection of pooled urine from five mice.

### Collection of Urine

Upon the last exposure to JP-8 or control vehicle, the mice were transferred to metabolic cages (Nalgene® Labware, Nalge Nunc International, Rochester, New York) to collect urine for 12- (Table 2) or 24-h periods (Tables 1 and 2). At this time, multiple mice from each treatment group were placed in each metabolic cage to ensure collection of adequate volumes of urine for MEAA analysis. It was our experience that a single mouse (*ca.* 20 g) would produce approximately 0.5 to 1.0 mL of urine during a 24-h period, which was not sufficient for multiple MEAA analysis requiring greater volumes of urine.

**TABLE 2**
Time course experiment after acute exposure (assay values as $\mu$g MEAA per mL urine)

| | Urine collection periods | | | |
|---|---|---|---|---|
| Description of Dose | 0–12 H | 12–24 H | 24–48 H | 48–72 H |
| | | A. Oral dose | | |
| Oil | nd | nd | nd | nd |
| 500 mg/kg | 2.8 | 0.6[1] | nd | nd |
| 1000 mg/kg | 4.5 | 2.0 | 0.4 | nd |
| 2000 mg/kg | 3.6[2] | 1.3 | 0.7 | nd |
| | | B. Dermal dose | | |
| Oil | nd | nd | nd | nd |
| 10 $\mu$L | 0.4 | 0.4 | nd | nd |
| 50 $\mu$L | 1.4 | 0.5 | nd | nd |
| 75 $\mu$L | 1.9 | 0.6 | nd | nd |
| | | C. control | | |
| No dose | nd | nd | nd | nd |

Notes:
nd = none detected.
All assay values are the average of two sample determinations except where noted (1 = only one assay due to low sample volume, 2 = average of three sample determinations), urine was collected and pooled from five mice.

## 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) used for the evaluation and integration of all raw data. The column type used was a capillary HP-1 (Agilent Technologies) with a length of 50 meters, internal diameter of 0.20 mm, and film thickness of 0.33 $\mu$m. The instrumental conditions for analysis were as follows: helium carrier flow was 0.8 mL/min constant, and injector port temperature was 240°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 postrun 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, selected ions were monitored at ion m/z 59 (MEAA) and ion m/z 66 (d-BAA) for quantification, and the detector source temperature was 230°C with the quadrupole set at 150°C. 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 volume of the final solution was 0.5 $\mu$L using splitless mode injection.

## Extraction and Esterification of MEAA from Mouse Urine

The extraction procedure was an adaptation of one described previously (B'Hymer et al. 2003; B'Hymer and Cheever 2004). Carefully measured volumes of mouse urine (0.5 to 2.0 mL) were added to a screw-capped culture tube and diluted to 4.0 mL with deionized water. This mixture was acidified with 20 $\mu$L of concentrated (12 M) hydrochloric acid. A 0.5-mL aliquot of 40 $\mu$g/mL of d-BAA internal standard solution was added. A 0.5-mL portion of deionized water 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.

## Standard Preparation

MEAA standards for calibration were prepared by adding 0.4, 1, 2, 4, 8, 20, 40, 80, 120, 160 and 200 $\mu$g of MEAA in a 0.5-mL aliquot of a standard spiking solution to a 4.0-mL sample

of human urine (mouse urine was not available in quantity and will be discussed later in this manuscript). A 0.5-mL aliquot of 40 $\mu$g/mL of d-BAA internal standard solution was added. This mixture was acidified with 20 $\mu$L of concentrated (12 M) hydrochloric acid and treated as described above with ethyl acetate extraction, esterification, and final methylene chloride extraction for GC analysis.

### Calculations

Calculations were based on peak area ratios of MEAA ethyl ester to d-BAA ethyl ester. Standard calibration curves were linear within the 0.4 to 200 $\mu$g per sample aliquot MEAA range used; correlation coefficients were 0.98 or greater and y-intercepts approached zero for all curves generated with this method. A complete calibration curve was run at the beginning and end of each batch of the collected urine. Calibration curve slope drift was minimal; less than 2% was observed within any batch run. Duplicate injections were performed for all samples and average values calculated for the reported data.

## RESULTS AND DISCUSSION

### Separation and Detection of MEAA Ethyl Ester

A chromatogram of the ethyl esters of MEAA and d-BAA extracted from spiked human urine is shown in Figure 2. The two peaks are easily resolved from one another and display no interfering peaks. The largest peak in the chromatogram was from the formation and extraction of the diethyl ester of sulfuric acid and had a retention time of approximately 19 min. The validation of the chromatographic procedure using spiked human urine has been described previously (B'Hymer et al. 2003). A total ion chromatogram of the ethyl esters of MEAA and d-BAA from a dosed mouse urine extract is shown in Figure 3. The mouse urine typically contained more peaks, but the selectivity of the

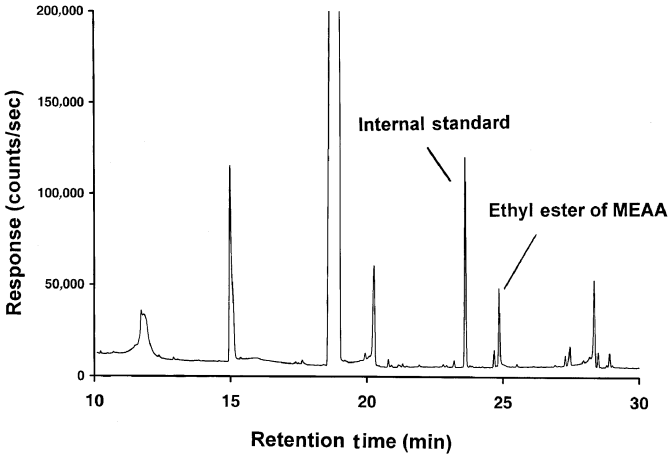


**FIG. 2.** A total ion chromatogram of a standard solution analyzed by the described procedure. This standard was prepared by spiking 20 $\mu$g of MEAA to 4 mL of urine and performing the extraction and esterification procedure. The internal standard is the extracted ethyl ester of d-butoxyacetic acid. The large peak at retention time 19 minutes was from the diethyl ester of sulfuric acid.

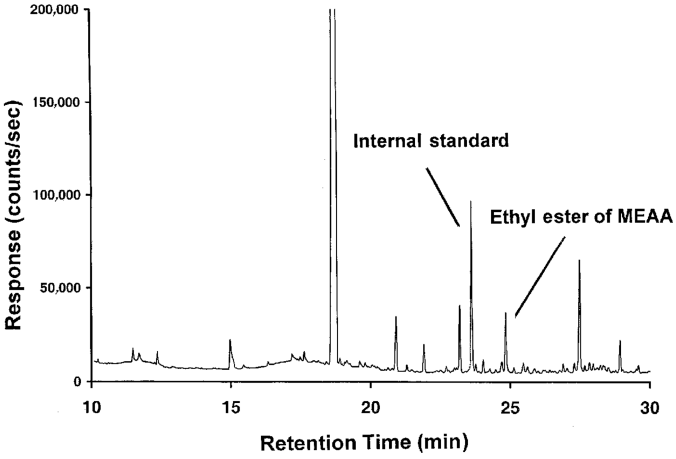


**FIG. 3.** A total ion chromatogram of a dosed mouse urine sample. MEAA was determined to be 8.5 $\mu$g/mL in this sample of urine.

MS detector from monitoring single characteristic ions eliminated the chromatographic complexity and maintained the validity of detecting and quantitating MEAA in the urine of dosed mice. This would seem to indicate that urine from both humans and mice is equivalent with respect to this test method for the chromatographic detection of the ethyl ester of MEAA. Chromatographic peak shape was excellent and there was no evidence of any carry-over between injections of samples; nonspiked human urine extract samples injected after the highest standard sample displayed no MEAA ethyl ester peak. (To further ensure that no carry-over occurred during sample runs, methylene chloride injections were placed between extract sample injections). The MEAA ethyl ester and d-BAA ethyl ester peaks were well resolved from each other with more than a 1-min separation in retention times. The nondrifting baseline on the gas chromatogram, which facilitates calculation of accurate peak areas, was obtained by using the gradual temperature program of only 3°C per minute. The limit of detection of MEAA ethyl ester was estimated to be approximately 0.2 ng per injection depending upon background noise level and the condition of the column used.

### Extraction of MEAA and Internal Standard from Urine

The use of ethyl acetate for the extraction of MEAA from urine was considered effective. It has been demonstrated that extraction efficiency of metabolic acids in urine approaches 80% when using ethyl acetate (B'Hymer and Cheever 2004). This method also utilizes a procedural internal standard, d-BAA. Variations in extraction yield, as well as the esterification step, would be similar for both compounds, thus normalizing the assay results. d-BAA is chemically similar to MEAA and has similar solubility and extraction properties. Additionally, the use of an internal standard increases the precision of the chromatographic injections. Relative standard deviations (RSD) of peak area ratios have ranged from 0.1 to 1.9% using this method (B'Hymer et al. 2003).

This test procedure measures total urinary MEAA. Since the ethyl acetate extraction of urine was performed under acidic conditions, and esterification of MEAA is under strong acidic conditions, many of the conjugated MEAA metabolites shown in Figure 1 are likely to be hydrolyzed to MEAA and converted to the ethyl ester along with the free urinary MEAA. Metabolite stability in urine and stability during the extraction and treatment conditions of the test procedure were not studied, owing to a lack of reference compounds for the metabolites of MEAA. This method should be considered an accurate measure for total urinary MEAA and, therefore, a reasonable measure of this biomarker for the exposure to jet fuel containing 2-(2-methoxyethoxy)ethanol.

## Procedural Recovery Studies of Spiked Mouse Urine Versus Human Urine Standards

The quantity of mouse urine was typically limited for these studies as mentioned previously. Because of its limited supply, human urine was ultimately chosen to be used in the preparation of standard samples. Eleven spiked samples plus a nonspiked and control standard were needed for each run; 2-mL volumes for each would require nearly 30 mL of control mouse urine per chromatographic run. Substitute urine was required in quantity. Artificial or synthetic urine (Kark et al. 1964) was one possible choice; however, it has been reported to have certain limitations (Downs and Perkins 2002; Greyer et al. 1995), making artificial urine not useful for these experiments. Human urine using 4-mL volumes was used and chromatographically caused no problems as described previously. The larger volume would increase the level of other extractables and be closer to the chromatographic complexity of mouse urine.

Matrix extraction differences between human and mouse urine probably were minimal, and these differences were demonstrated by spiked recovery experiments. Extensive recovery studies of spiked human urine samples using this procedure have been reported previously (B'Hymer et al. 2003). This earlier work demonstrated that this method was capable of 95 to 103% recovery with 2 to 20 $\mu$g/mL spiked human urine with relative standard deviation of less than 7.8%. This earlier validation study did not include any applications of in vivo urine samples. Although the limited supply of urine from mice has been a factor, spiked studies thus far have given full recovery. Mouse urine fortified with 10 $\mu$g/mL MEAA demonstrated a recovery of 99% (n = 3) for the first test day and 108% (n = 3) for the second test day during the animal testing runs. This represented 104% recovery (10.4 $\mu$g/mL, n = 6, relative standard deviation [RSD] = 7.1%) for the separate spiked mouse urine samples and was within the statistical precision of the test procedure. Any sample bias between the mouse urine extract and the standards was not apparent, or at least, it was statistically insignificant. The precision level, RSD = 7.1%, obtained from these spiked mouse urine samples is similar to the levels obtained from spiked human urine samples (B'Hymer et al. 2003). More spiked mouse urine sample recovery studies are planned as future work.

## Animal Data

Pilot studies using oral or dermal exposure routes in B6C3F1 mice were performed to identify the utility of this method for use in immunotoxicology studies. B6C3F1 female mice are standard to immunotoxicology studies and were used in this pilot because this laboratory has previously determined a profile of immunological dysfunction that corresponds with a week-long exposure to JP-8. It was desirable to establish markers of exposure that are applicable to laboratory species as well as in exposed humans; thus, the impetus of this study was to establish that MEAA was a quantifiable urinary metabolite in a laboratory species exposed to JP-8. These data provided are preliminary in nature, but unequivocally demonstrate that MEAA is quantifiable in mouse urine after two routes of exposure. Table 1 displays data collected after 1 week daily exposure; that is, urine was collected during a period of 24 h following 7 consecutive days of exposure to either dermal or oral dosages of JP-8. MEAA was not detected in urine of control mice treated with oil or acetone, while urine collected from mice dosed orally at 2000 mg/kg or having 50 $\mu$L dermal applications had average levels of 8.5 or 6.5 $\mu$g/mL MEAA. Table 2 represents a time course study evaluating the levels of MEAA after a single exposure to JP-8. By 72-h, MEAA was not detected after oral exposure at all treatment levels, while MEAA was not detectable by 48 h in all dermally exposed mice. In general, this time course study demonstrates that increased levels of exposure to JP-8 correlate with increased levels of urinary MEAA. However, average levels of MEAA at the 12- and 24-h time points in 2000 mg/kg orally dosed mice are less when compared to 1000 mg/kg exposed mice. This may be attributed to variability associated with the small sampling group, as urine was pooled from only five group-housed mice. It might also be inferred that the mice exposed to the higher quantities of JP-8 had inhibited liver or metabolic function, possibly due to other components of JP-8 confounding hepatic metabolic function. Some other glycol ethers have been observed to have lower acid metabolites after increased dosages; the relative abundance of butoxyacetic acid decreases with increasing the dose of 2-butoxyethanol (Medinsky et al. 1990). Also, it has been suggested that the metabolic pathway by alcohol dehydrogenase (ADH) can become saturated by some glycol ethers (Aasmoe et al. 1998). Although intriguing, these data represent preliminary work and the focus of this manuscript is on the validation of a method for the quantification of urinary MEAA as applied to mice. Therefore, conclusions should be withheld about the metabolism of JP-8 or MEAA in the mouse and are beyond the scope of this manuscript since this was only a pilot animal study. More importantly, these data do demonstrate the usefulness of the adapted procedure to detect and quantify the levels of MEAA in mouse urine. 2-(2-Methoxyethoxy)ethanol is a common, fixed component in all mixtures of JP-8 fuel; therefore, total urinary MEAA was demonstrated to be a viable biomarker for mice exposed to JP-8.

## Future Considerations

Future work at this laboratory will include much more extensive use of this test procedure to support toxicological studies using mice. The accurate and precise determination of MEAA in urine is required for the planned immunotoxicological studies. The dermal toxicity of 2-(2-methoxyethoxy)ethanol needs to be better defined, and the utility of MEAA as a biomarker of JP-8 exposure must be further evaluated. The toxicity of MEAA, the acid metabolite itself, has not been completely evaluated in humans and will undoubtedly be another aspect of research in the future. Also, the metabolism of some glycol ethers have exhibited sex-related differences in toxicological responses in animals (Aasmoe and Aarbakke 1997; Aasmoe et al. 1998), and may become another area of research.

In summary, a procedure has been devised to detect and quantify (2-methylethyl)acetic acid (MEAA) in urine collected from mice. Preliminary studies using mice exposed orally or dermally to JP-8 jet fuel resulted in the detection of MEAA in the collected urine at levels as high as 8.5 $\mu$g/mL from the oral exposure study and 6.5 $\mu$g/mL in the dermal study. This test procedure should be applicable to future toxicological studies of mice. With this study confirming that urinary MEAA is quantifiable in mice, novel links between toxicological rodent models and human exposure can explored. More importantly, total urinary MEAA may serve as a marker of exposure to 2-(2-methoxyethoxy)ethanol, a specific fixed component in the formulation of JP-8.

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