Article

Development of an Analytical Method for Quantitation of 2,2'-Dimorpholinodiethyl Ether (DMDEE) in Rat Plasma, Amniotic Fluid and Fetal Homogenate by UPLC–MS–MS for Determination of Gestational and Lactational Transfer in Rats

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Abstract

2,2'-Dimorpholinodiethyl ether (DMDEE) is a specialty amine catalyst used in the production of flexible foams, adhesives and coatings. The potential for occupational exposure to DMDEE is high, but toxicity data are very limited. The objective of this work was to develop a method to quantitate DMDEE in biological matrices to assess gestational and lactational transfer of DMDEE in rats following exposure of dams. The method used protein precipitation, followed by removal of phospholipids and analysis of supernatant by ultra-performance liquid chromatography-tandem mass spectrometry. Rat fetuses were homogenized in water prior to protein precipitation and delipidation procedures. The method was evaluated in male Sprague Dawley rat plasma over the concentration range 5 to 1000 ng/mL. The method was linear ($r \geq 0.99$), accurate (mean relative error (RE) $\leq \pm 11.9\%$) and precise (relative standard deviation (RSD) $\leq 2.7\%$). The mean absolute recovery was 106%. The limit of detection was 0.262 ng/mL. Standards as high as ~100,000 ng/mL could be successfully diluted into the calibration range (mean %RE = -14.9; %RSD = 0.5). The method was evaluated in Sprague Dawley rat dam plasma, post-natal day 4 pup plasma, gestational day (GD) 18 amniotic fluid and fetal homogenate (mean %RE $\leq \pm 11.9$; %RSD ≤ 2.3). Concentrations of DMDEE in rat dam plasma, amniotic fluid and fetal homogenate stored for at least 29 days and in pup plasma for at least 18 days at -80°C were within 87.7 to 99.5% of Day 0 concentrations, demonstrating that DMDEE is stable in these matrices. The method was used to quantitate DMDEE in rat plasma, amniotic fluid and fetus samples from a dose range finding toxicology study in which dams were dosed via gavage with DMDEE from GD 6 at doses of 0 (control), 62.5 and 250 mg/kg/day. DMDEE concentration increased with the dose in all matrices examined. The concentration in GD 18 fetuses was almost 2-fold higher than GD 18 dams demonstrating gestational transfer of DMDEE. However, the concentration in post-natal day 4 pup plasma was

more than an order of magnitude lower than corresponding dam plasma suggesting less potential for transfer of DMDEE from dams to pups via lactation. There was no significant difference in concentration for male and female pup plasma.

Introduction

2,2'-Dimorpholinodiethyl ether (DMDEE) is a blowing agent used in the production of flexible, molded and moisture-cured foams, coatings and adhesives (1). Production of DMDEE was reported between 1 and 10 million pounds in the USA in 2013 (2). The potential for occupational exposure to DMDEE is high; workers may be exposed to vapors emitted from cutting foam, during application of hot melt adhesives at elevated temperatures, or to unreacted catalyst during final production (3). There is concern that *N*-nitrosomorpholine, a well-known hepatocarcinogen, could be a metabolite of DMDEE (4).

No toxicity data are available for DMDEE. Hence, the National Toxicology Program is evaluating the toxicology of DMDEE (<https://ntp.niehs.nih.gov/testing/status/agents/ts-m060065.html>). Quantitative determination of internal dose after DMDEE exposure is essential in interpreting toxicological outcome. Limited methods have been published for determination of DMDEE in polyurethane materials by high-performance liquid chromatography-mass spectrometry (HPLC-MS) (5), direct analysis in real time mass spectrometry (DART-MS) (6) and pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) (7). However, to the best of our knowledge, there are no methods for the quantitation of DMDEE in biological matrices. To support the proposed toxicology studies investigating maternal transfer of DMDEE, a simple yet sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) method was developed in rat dam plasma, pup plasma, amniotic fluid and fetal homogenate. Samples from a dose range finding study were analyzed to evaluate the feasibility of the method and for initial assessment of maternal transfer of DMDEE.

Experimental Design

Chemicals and reagents

DMDEE (99.0% purity) was purchased from Gojira Fine Chemicals (Bedford Heights, OH), and 4-benzoylmorpholine (internal standard, IS) and 4-[2-(piperazin-1-yl)-acetyl]-morpholine were from Combi-Blocks (San Diego, CA). Bis(4-morpholinyl)methane was from Alfa Aesar (Tewksbury, MA), 1,1-bis(morpholino)ethylene was from TCI America (Portland, OR), 1,3-dimorpholyl-2-propanol was from MP Biomedicals (Santa Ana, CA) and propranolol was from Sigma-Aldrich (St. Louis, MO). Adult male and post-natal day (PND) 4 pup Sprague Dawley (SD) rat plasma (isolated from blood collected with K₃EDTA anticoagulant) and gestational day (GD) 18 fetuses were purchased from BioIVT (Westbury, NY). GD 18 SD rat dam plasma and amniotic fluid were purchased from Hilltop Lab Animals (Scottsdale, PA). HPLC- and LC-MS-grade solvents were purchased from commercial sources.

Method development and evaluation

GC-MS and LC-MS-MS methodologies were investigated with respect to sample preparation, chromatographic performance, sensitivity and dynamic range. The final UPLC-MS-MS method was

evaluated in adult male SD rat plasma (primary matrix) by analyzing calibration standards and quality control (QC) samples in replicates to demonstrate linearity, selectivity, sensitivity, precision and accuracy over the concentration range 5–1000 ng/mL. Selectivity was assessed by analyzing triplicate matrix blanks with and without IS for background interferences. Accuracy was evaluated as percent relative error (%RE) and precision as relative standard deviation (RSD). The lower limit of quantitation (LLOQ) was defined as the lowest standard that could be accurately quantitated within 20% of the nominal value and at which 6 replicates could be reproduced within 20% RSD. The limit of detection (LOD) was defined as three times the standard deviation of the LLOQ response expressed as concentration. Recovery was expressed as the percentage of the response of the analyte in matrix to that in solvent. Precision and accuracy were evaluated at two concentration levels (15 and 500 ng/mL) using three independent standards prepared on the same day. The method was also evaluated in secondary matrices: SD dam plasma, GD 18 amniotic fluid, GD 18 fetal homogenate and PND 4 pup plasma.

Preparation of stock solutions

Standard stock solutions of DMDEE were prepared at 250 and 500 µg/mL in water. Spiking solutions of DMDEE (25 to 5000 ng/mL) were prepared in water from the alternating DMDEE stock solutions and were used to prepare the matrix calibration standards and QC samples. A stock solution of the IS was prepared at 200 µg/mL in water and was diluted to prepare a working solution at 500 ng/mL in water. Standard solutions were stored refrigerated (~5°C) when not in use.

Preparation of primary matrix standards and blanks

Plasma calibration standards were prepared by fortifying 25 µL of blank male SD rat plasma with 5 µL of the appropriate spiking standard to obtain plasma DMDEE concentrations of 5, 15, 100, 250, 500 and 1000 ng/mL. QC samples were prepared similarly at 15 and 500 ng/mL. For matrix blanks, 5 µL of water were substituted for the spiking solution. Solvent standards were prepared at 3 concentrations (5, 100 and 1000 ng/mL) in the same manner as the matrix standards, except 25 µL of water were used instead of rat plasma. A 5-µL aliquot of the IS working solution was added to each sample. Sample extraction was achieved by addition of 500 µL of acetone, vortex mixing and freezing at –80°C for 30 minutes to separate the phospholipids. After centrifugation at 12,000 rpm for 10 minutes at 0°C, the supernatant was removed and diluted 1:1 with water for UPLC-MS-MS analysis. Dilution verification was conducted at ~100,000 ng/mL in male rat plasma. The samples were extracted as mentioned earlier, diluted 100-fold with 50:50 acetone:water containing 5 ng/mL IS working solution, and then diluted 1:1 with water for UPLC-MS-MS analysis. Figure 1 is a schematic showing the preparation of matrix standards.

Figure 1. Schematic of sample preparation method.

Secondary matrix evaluation in GD 18 dam plasma amniotic, fluid fetal homogenate and PND 4 pup plasma

SD fetal homogenate was prepared by mechanical disruption (SPEX Geno/Grinder 2010, Metuchen NJ) with 2.3 mm stainless steel beads in a 1:1 volume of chilled water (1 mL water per 1 g of fetus). Triplicate matrix standards containing DMDEE were prepared at two concentrations (15 and 500 ng/mL) in each secondary matrix and analyzed in the same manner as primary (male rat plasma) matrix standards. The secondary matrix standards were quantitated using a curve prepared in the primary matrix. Triplicate matrix

blanks containing IS were prepared in each matrix to evaluate selectivity.

Stability

Analyte stability in matrix during storage at -80°C was established for sets of secondary matrix samples (GD 18 dam rat plasma, amniotic fluid, fetal homogenate and PND 4 pup plasma) prepared at 15 ng/mL in triplicate and evaluated at predetermined intervals up to 30 days to cover the anticipated storage period for study samples. Results were evaluated by comparing data to triplicate fresh (Day 0) samples.

UPLC–MS–MS analysis

The UPLC–MS–MS system consisted of a Waters Acquity UPLC (Milford, MA) coupled to a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray (electrospray) source (Sciex, Framingham, MA). Chromatographic separation was performed using a Waters Acquity UPLC BEH C18 column (50 mm $\times$ 2.1 mm i.d., 1.7 μm particle size) and a Waters Acquity UPLC BEH C18 guard column (5 mm $\times$ 2.1 mm i.d., 1.7 μm particle size). Two microliters of prepared sample were injected onto the column and elution was achieved at 30°C using a binary gradient and a flow rate of 0.3 mL/min. The needle washes consisted of 10% acetonitrile in water (weak needle wash) and 40:30:30 methanol:water:acetone with 2% formic acid (strong needle wash) to eliminate injection carryover. The mobile phases consisted of (A) 2.5 mM ammonium bicarbonate in water and (B) acetonitrile. The gradient was 5% mobile phase B for 0.5 minute, 5 to 95% B from 0.5 to 3.5 minutes, hold at 95% B for 0.5 minute. The electrospray ion source was operated in positive ion mode with an ion spray voltage of 5,500 V and source temperature of 700°C . The collision gas was 12 psi, the curtain gas was 40 psi, the nebulizer gas was 30 psi, the auxiliary gas was 70 psi and the interface heater was on. The selected multiple reaction monitoring (MRM) ion transitions were $245.2 \rightarrow 114.1$ for DMDEE, and $192.2 \rightarrow 105.1$ for the IS. The optimized compound-dependent parameters for DMDEE were declustering potential = 56 V; entrance potential = 10 V; collision energy = 29 V and collision cell exit potential = 19 V, and for the IS: declustering potential = 46 V; entrance potential = 10 V; collision energy = 23 V and collision cell exit potential = 18 V. Analyst software version 1.6.2 (Sciex) was used for data acquisition and analysis.

Calibration curves were generated by plotting the peak area ratios of DMDEE to IS as a function of analyte concentration. The regression model for DMDEE was a linear weighted least squares algorithm with a weighting factor of $1/\text{concentration-squared}$ ($1/x^2$). The concentration of DMDEE was calculated using response ratio, the regression equation, initial sample volume and dilution when applicable. Plasma and amniotic fluid data are expressed as ng/mL, while fetus data are expressed as ng/g fetus.

Animal studies

Exposures to animals were conducted at Southern Research (Birmingham, AL). All procedures that involved the use of animals were approved by Southern Research's Institutional Animal Care and Use Committee. Time-mated, $\sim$ GD 2, female Hsd:Sprague Dawley[®]SD rats (HSD; age 12–14 weeks; $\sim$ 200–225 g) were purchased from Harlan Laboratories (Haslett, MI) and housed individually. They were provided tap water (Birmingham, AL) and control feed ad

libitum (irradiated and certified NIH-07 wafer diet, Ziegler Bros, Gardners, PA). Prior to administration of DMDEE, female rats were randomized into treatment groups using GD 3 body weights and uniquely identified. Pups were identified by paw tattoo according to number within litter at PND 1. Dams were housed one per cage and pups remained with their respective dam until euthanasia or weaning on PND 28.

For collection of biosamples, time-mated female rats/group were exposed to DMDEE by oral gavage at 0 (control), 62.5 and 250 mg/kg/day beginning on GD 6; exposure continued throughout gestation and lactation until PND 4. Dam plasma, amniotic fluid (pooled by litter) and fetuses (pooled by litter) from 3 dams per group were collected at GD 18, and dam and pup (both male and female) plasma samples from another 3 dams per group were collected at PND 4. Sample collection started ~2 hours after the last dose and was collected within a ~2-hour window to minimize variability. Blood samples were collected via cardiac puncture into tubes containing K₃EDTA anticoagulant and plasma was prepared by centrifugation at 4°C. All samples were snap-frozen in liquid nitrogen upon collection, then stored at ≤−70°C until shipment on dry ice. Once received in the analytical laboratory, samples were stored at ≤−70°C until analysis.

Study samples were prepared and analyzed similar to above except no DMDEE was added. Two fetuses were randomly selected from each litter for analysis. A 6-point matrix calibration curve from 5 to 1000 ng/mL was run at the beginning of each sample run, and interspersed QCs were run at 2 levels (15 and 500 ng/mL). A successful calibration was indicated by $r \geq 0.99$ and $RE \leq 15\%$ for all calibration standards (except at LLOQ $RE \leq 20\%$) and at least 67% of the QCs in each run.

Results and Discussion

Method development

No methods for the analysis of DMDEE in biological matrices were found in the literature. GC-MS method development was initiated, but peak shape was poor due to the basicity of DMDEE and a time-consuming extraction method involving solid phase extraction or liquid-liquid extraction was required. A UPLC-MS-MS method was thus developed, and different column chemistries and mobile phases were explored to optimize analyte retention and peak shape. A BEH C18 column was selected because it was stable under the basic conditions (pH 8) needed for analyte retention.

In the absence of a commercially available stable-isotope-labeled standard, six different compounds were considered for IS: bis(4-morpholinyl)methane; 1,1-bis(morpholino)ethylene; 4-[2-(piperazin-1-yl)-acetyl]-morpholine; 1,3-dimorpholyl-2-propanol; propranolol and 4-benzoylmorpholine. Based on peak shape, recovery and sensitivity, 4-benzoylmorpholine was selected as the most suitable IS. Structures of DMDEE and 4-benzoylmorpholine are shown in Figure 2.

During method development, sources of excessive background were identified in the buffer reagents, possibly a coating or adhesive. Using ammonium bicarbonate from a glass bottle minimized the background. High background was also observed when the mobile phase was filtered through a GH Polypro membrane. Analyte carryover and subsequent background were issues throughout method development and prevented extension of the calibration curve beyond ~5–1000 ng/mL.

Using a simple acetonitrile protein crash, relative recoveries for DMDEE were >100%, indicating possible matrix effects. These

Figure 2. Structures of DMDEE (top) and 4-Benzoylmorpholine (IS, bottom).

effects were minimized with methanol or acetone protein precipitation followed by freezing (−80°C for 30 minutes) to separate the phospholipids, as developed previously in our laboratory (8). Acetone gave the most consistent results and was therefore selected as the extraction solvent. Sample amounts were minimized; only 25 µL of plasma, amniotic fluid or fetal homogenate was required. The final method requires only a single-step protein precipitation for plasma and amniotic fluid, and simple extraction and delipidation of fetal homogenate.

Method evaluation

No interfering peaks were detected in the matrix blanks, indicating that the method was selective for DMDEE. Representative chromatograms are presented in Figure 3. The method was evaluated

Figure 3. Representative UPLC-MS-MS chromatograms for DMDEE matrix standards: blank rat plasma with IS, 15 ng/mL DMDEE in plasma and 1000 ng/mL DMDEE in plasma.

Table I. Method Evaluation Data for DMDEE

Parameter	Result
Nominal matrix concentration range (ng/mL)	5.18–1010
LOD ^a (ng/mL)	0.262
LLOQ ^b (ng/mL)	5.18
Precision (%RSD) ^c	1.6
Accuracy (mean %RE) ^d	5.2
Correlation coefficient (<i>r</i>)	≥0.99
Mean absolute recovery (%)	106
Precision and Accuracy ^e	
Intra-assay precision (%RSD)	≤2.7
Intra-assay accuracy (mean %RE)	≤±11.9
Dilution verification ^f	
Precision (%RSD)	0.5
Accuracy (mean %RE)	−14.9
Secondary matrix evaluation ^g	
Precision (%RSD)	
GD 18 dam plasma	≤2.0
GD 18 amniotic fluid	≤2.3
GD 18 fetal homogenate	≤2.0
PND 4 pup plasma	≤1.5
Accuracy (mean %RE)	
GD 18 dam plasma	≤±11.9
GD 18 amniotic fluid	≤±9.1
GD 18 fetal homogenate	≤±11.9
PND 4 pup plasma	≤±10.8

^aLOD = limit of detection.^bLLOQ = lower limit of quantitation; lowest standard at which RE ≤ ±20% and RSD ≤ 20% for *n* = 6.^c%RSD = percent relative deviation.^d%RE = percent relative error.^ePrecision and accuracy determined for triplicate QCs at 2 levels: 15.1 and 518 ng/mL.^fPrecision and accuracy determined for triplicate QCs at 99,500 ng/mL.^gPrecision and accuracy determined for triplicate QCs at 2 levels in each secondary matrix: 15.1 and 518 ng/mL.

using male rat plasma (primary matrix) to demonstrate linearity, sensitivity, accuracy, precision, dilution integrity and stability; method evaluation data are summarized in Table I. The calibration curves were linear with correlation coefficients $r \geq 0.99$. Precision was ≤2.7% RSD, and accuracy was ≤±11.9% RE over the calibration range. The LLOQ was 5.18 ng/mL (mean %RE = 5.2%; 1.6% RSD). The LOD, defined as three times the standard deviation of the LLOQ response, was 0.262 ng/mL. The mean absolute recovery was 106% (1.6% RSD). Plasma samples prepared at 99,500 ng/mL, extracted and diluted into the calibration range, were found to be acceptable with mean RE = −14.9% and 0.5% RSD.

QCs and blanks prepared in secondary matrices (SD rat GD 18 dam plasma, amniotic fluid, fetal homogenate, and PND 4 pup plasma) were quantitated using a male SD rat plasma curve (primary matrix). Again, no interfering peaks were detected in the secondary matrix blanks, indicating that the method was selective for DMDEE. For each secondary matrix, precision was ≤2.3% RSD, and accuracy was ≤±11.9% RE.

Stability

Studies were performed to evaluate the stability of DMDEE in the secondary matrices at −80°C for a period of time approximately equal to the duration of the study sample storage (18–29 days). The determined concentrations were compared to those of freshly prepared samples ('Day 0'), and data are presented in Table II. DMDEE concentrations were within 87.7 to 99.5% of day 0 (RSD ≤ 3.5%),

Table II. Matrix Stability^a

Endpoint	Mean % of Day 0 (%RSD)
Dam plasma (29 d)	93.1 (1.3%)
Amniotic fluid (29 d)	93.6 (2.2%)
Fetal homogenate (29 d)	99.5 (3.5%)
Pup plasma (18 d)	87.7 (0.9%)

^aDetermined for triplicate QCs at 15.1 ng/mL and stored at −80°C.

Figure 4. Representative UPLC-MS-MS chromatograms for DMDEE in PND 4 dam plasma, PND 4 pup plasma, GD 18 amniotic fluid and GD 18 fetal homogenate following gavage administration of 62.5 mg/kg/day DMDEE in pregnant rats starting on GD 6.

demonstrating stability of DMDEE in matrices under these conditions and durations.

Study sample analysis

The method was successfully applied to quantitate DMDEE concentration in HSD GD 18 dam plasma, amniotic fluid, fetuses, and PND 4 dam and pup plasma in a dose range finding toxicology study where dams were exposed to DMDEE via gavage at 0 (control), 62.5 and 250 mg/kg/day. DMDEE was measured in all matrices in exposed groups at concentrations above the LOD of the assay. Concentrations of DMDEE in control samples were below the LOD of the assay. Example chromatograms are presented in Figure 4, and the concentrations of DMDEE for each dose group in each sample type are given in Table III and Figure 5. DMDEE concentration in all matrices increased linearly with the dose. At a given dose, the DMDEE concentration was almost 2-fold higher in GD 18 fetus than in dam plasma showing significant transfer of DMDEE from dam to

Table III. Concentrations of DMDEE in Plasma, Amniotic Fluid and Fetuses following Daily Gavage Administration of DMDEE to Pregnant Rats Starting on GD 6^a

Dose level (mg/kg/day)	GD 18 dam plasma (ng/mL)	GD 18 amniotic fluid (ng/mL)	GD 18 fetus (ng/g)	PND 4 dam plasma (ng/mL)	PND 4 male pup plasma (ng/mL)	PND 4 female pup plasma (ng/mL)
0	ND ^b	ND	ND	ND	ND	ND
62.5	22,600 (2,110)	14,300 (1,310)	42,800 (2,490)	21,500 (2,490)	697 (148)	729 (101)
250	71,900 (8,500)	60,400 (4,740)	121,000 (9,810)	53,000 (5,570)	2,640 (412)	2,350 (472)

^aMean (standard error) for $n = 3$ animals are given.^bND, not detected. Concentration was below LLOQ (~ 5 ng/mL) of the assay. **Figure 5.** Mean concentrations of DMDEE in plasma, amniotic fluid and fetuses following gavage administration of DMDEE in pregnant rats starting on GD 6. Error bars show standard error of the mean for $n = 3$ animals. The change in scale for the y-axis is marked by a line break.

fetus. However, the concentration in PND 4 pup plasma was more than an order of magnitude lower than corresponding dam plasma suggesting less potential for transfer of DMDEE to pups via lactation. There was no significant difference in concentration for male and female pup plasma. To the best of our knowledge, this is the first study reporting internal exposure data and maternal transfer data for DMDEE.

Conclusions

A rapid, sensitive UPLC–MS–MS method was developed to quantitate DMDEE in rat plasma, amniotic fluid, and fetal homogenate over the range 5–1000 ng/mL, with the ability to dilute samples up to 100,000 ng/mL into range. The method requires only 25 μ L of sample and extraction with acetone followed by freezing to separate the phospholipids. The method was used to quantitate DMDEE in plasma, amniotic fluid and fetus samples from a dose range finding toxicology study in which dams were dosed orally with DMDEE from GD 6 to PND 4. DMDEE was found in all matrices in exposed groups suggesting fetal and lactational transfer of DMDEE in rats following exposure of dams this method could be validated to support additional future toxicokinetic or toxicology studies of DMDEE.

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