Quantitative Determination of the Hydrolysis Products of Nitrogen Mustards in Human Urine by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry*

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Abstract

Nitrogen mustards are a public health concern because of their extreme vesicant properties and the possible exposure of workers during the destruction of chemical stockpiles. A sensitive, rapid, accurate, and precise analysis for the quantitation of ultratrace levels of N-ethyldiethanolamine (EDEA) and N-methyldiethanolamine (MDEA) in human urine as a means of assessing recent exposure to the nitrogen mustards bis(2-chloroethyl)ethylamine and bis(2-chloroethyl)methylamine, respectively, was developed. The method was based on solid-phase extraction, followed by analysis of the urine extract using isotope-dilution high-performance liquid chromatography–mass spectrometry with TurbolonSpray® ionization and multiple-reaction monitoring. The method limits of detection were 0.41 ng/ml for EDEA and 0.96 ng/ml for MDEA in 1 mL of urine with coefficients of variation < 10% for both compounds.

Introduction

As part of the preparedness and response efforts to a terrorist attack, the Centers for Disease Control and Prevention have been developing analytical methods to measure biomarkers of exposure to chemicals that have potential use as chemical terrorist agents. The nitrogen mustards bis(2-chloroethyl)ethylamine (HN1) and bis(2-chloroethyl)methylamine (HN2) represent two such chemicals that are listed on the Chemical Weapons Convention Schedule of Chemicals (1). Like sulfur mustards, nitrogen mustards possess strong vesicant properties, but nitrogen mustards are less suitable for military purposes (2).

Methods have been reported for the identification of nitrogen mustards in blood and plasma (3–5). However, the suitability of these methods for biomonitoring purposes is limited by the reactivity and extent of metabolism of the mustards (6). For example, one study showed that the blood concentration of HN2 peaks within 5 min of subcutaneous injection with an average half-life of 15 min (5).

HN1 and HN2 are bifunctional alkylating agents that readily react with biomolecules, such as DNA and proteins, to form nitrogen mustard adducts. Analogous to sulfur mustard adducts (7–9), nitrogen mustard adducts can be used as biomarkers of nitrogen mustard exposure. In particular, the reaction products between DNA and HN2 have been studied in vitro (10–16), and the major DNA-HN2 adduct, N-[2-(hydroxyethyl)-N-(2-(7-guaninyl)ethyl)]methylamine, has been quantitated by high-performance liquid chromatography (HPLC) with ultraviolet detection (12). However, the usefulness of these methods for in vivo studies has yet to be determined. Other possible biomarkers of exposure to the nitrogen mustards are the depurination products found in urine, such as the N-7-alkylguanines (N-7-G), formed as a result of the degradation and repair of nitrogen mustard-DNA adducts. Methods to determine endogenous N-7-Gs present in human urine have been reported in the literature (17–19). However, a drawback of using the N-7-Gs specific to the nitrogen mustards to quantitate the amount of nitrogen mustard exposure based on DNA adduct formation is the substantial individual variations in DNA-adduct formation (20) and repair.

HN1 and HN2 hydrolyze to N-ethyldiethanolamine (EDEA) and N-methyldiethanolamine (MDEA), respectively (21). Alkanolamines, such as EDEA and MDEA, have many legitimate industrial and commercial uses; consequently, several HPLC–mass spectrometry (MS) methods have been reported for the determination of these compounds in environmental samples (22–24). EDEA was measured in aqueous solution at relatively high concentration (160 μg/mL) using HPLC with postcolumn derivatization and MS with atmospheric pressure chemical
ionization (APCI) (22). Although derivatization shifted the EDEA [M + H]⁺ ion to a higher mass, possibly alleviating interferences with lower mass ions, the sensitivity of the method was not improved over the underivatized compound. Other researchers reported qualitative screening methods for EDEA and MDEA, among the hydrolysis products of several other CWAs, in aqueous extracts using HPLC–MS with positive ion APCI (23,24). Using single ion monitoring, EDEA and MDEA were detected at < 10 ng/mL with a 20-μL injection volume.

The only report we found relative to the determination of MDEA in biological specimens measured MDEA in the urine of rats 24 and 48 h after exposure to HN1 and HN2, and MDEA in human urine. The rapid and sensitive method compared to free EDEA or MDEA.

Furthermore, the analysis of the rat urines showed no increase in the concentration of free EDEA or MDEA after urine samples from the same animals were subjected to β-glucuronidase hydrolysis. These presently unpublished results suggest that, in urine, glucuronidated EDEA and MDEA are minor species compared to free EDEA or MDEA.

This paper describes the first quantitative analysis of EDEA and MDEA in human urine. The rapid and sensitive method used a simple solid-phase extraction (SPE) clean-up followed by HPLC–positive ion electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS–MS). The reproducibility of the method was enhanced by using isotopically labeled analogues as reference compounds.

Experimental

Materials

EDEA and MDEA were purchased from Aldrich Chemical Co. (Milwaukee, WI) at the highest commercially available purity; EDEA-[13C]4 and MDEA-[13C]4 were not commercially available and were custom synthesized by Cambridge Isotope Laboratories, Inc. (Andover, MA). Ammonium hydroxide solution (28–30%) was purchased from EM Science (Darmstadt, Germany); organic-pure water (OP H2O, Type I) was prepared using a Solution 2000™ water purification system (Solution Consultants Inc., Jasper, GA). All other solvents were HPLC grade and were used without further purification. Polypropylene labware was used as received and was found to improve recoveries over glassware without contributing to interferences.

Standards preparation and characterization

Native standards. A combined EDEA and MDEA 20-mg/mL stock solution was prepared by weighing approximately 500 mg of each compound into 25 mL of 15mM ammonium hydroxide. Serial dilutions of this stock solution were made in 15mM ammonium hydroxide to create eight standard solutions, each with 1 mL of urine resulted in the desired EDEA and MDEA concentrations (i.e., from 1.6 ng/mL to 270 ng/mL). Each stock and diluted solutions were flame-sealed into ampoules and stored at 4°C and at room temperature, respectively.

Labeled standards. A combined EDEA-[13C]4 and MDEA-[13C]4 1-μg/mL stock reference solution was prepared by pipetting 25 mL of each compound into 25 mL of 15mM ammonium hydroxide. A working labeled reference solution was prepared by diluting the stock solution in 15mM ammonium hydroxide, so that a 20-μL aliquot of the working solution in 1 mL urine resulted in an approximate 25 ng/mL concentration of each labeled compound. Stock and working labeled reference solutions were flame-sealed into ampoules and stored at 4°C and at room temperature, respectively.

Quality control (QC) materials. QC materials were prepared from a urine pool collected from multiple donors. A stainless steel pressurized dispenser was used to filter the urine through a 0.2-μm filter. The filtered urine was homogenized overnight and divided into three aliquots. Two aliquots were enriched with approximately equal amounts of EDEA and MDEA at two levels to create low (~10 ng/mL) and high (~250 ng/mL) QC materials. The third aliquot, representing the natural concentrations of EDEA and MDEA, was used as the method blank and the standard matrix. Enriched pools were thoroughly mixed and dispensed in 2-μL aliquots into precleaned 4-mL vials and stored at −20°C. Similarly, the blank QC was dispensed in 5-μL aliquots into precleaned 10-mL vials and stored at −20°C until needed. Each QC material was characterized by at least 20 analyses to define the mean concentrations and the 95th and 99th confidence intervals of EDEA and MDEA. QC materials reanalyzed after the initial characterization showed that EDEA and MDEA remained stable in the QC materials at −20°C for at least one year. Additionally, storage at −20°C was sufficient to prevent bacterial growth during this time.

Analyte extraction

One milliliter of urine was placed into a 15-mL conical polypropylene centrifuge tube and spiked with 20 μL of working labeled reference solution, then diluted with 1 mL of OP H2O (Type I) to reduce the ionic strength of the sample. For the standards, a 50-μL aliquot of the appropriate standard solution was added to the urine. The diluted urine sample was applied to a preconditioned 3-mL SPE column (LiChrolut, EM Separations, Darmstadt, Germany) containing 500 mg of a strong cation exchanger and allowed to pass through by gravity. The column was then washed with 3 mL of acetonitrile to remove extraneous compounds. EDEA and MDEA were eluted from the SPE column directly into 15-mL conical polypropylene centrifuge tubes using 5 mL of a 98% acetonitrile/2% ammonium hydroxide solution. Residual EDEA and MDEA were eluted by drying the SPE cartridge (15 mm Hg). The solvent was completely removed from the extracted sample using a TurboVap LV evaporator (Zymark Corp., Hopkinton, MA) at 70°C under 15 psi nitrogen. The sample was reconstituted in 0.5 mL of 3mM ammonium hydroxide and transferred into 2-mL polypropylene autosampler vials. Samples were processed
simultaneously through both the SPE procedure, which required approximately 1.5 h, and the 15-min evaporation procedure. The time required for individual sample manipulations such as transfers and solvent and standard additions was approximately 5 min per sample, so that sample processing for a typical 24-sample analytical run comprising QC materials, standards, and samples required approximately 4 h prior to HPLC–MS–MS analysis. The analytes were stable in the 3mM ammonium hydroxide extract for at least four weeks at 4°C.

Chromatography and MS

**Chromatographic conditions.** An Agilent 1100 LC system consisting of a binary pump, autosampler, column heater, and vacuum degasser (Agilent Technologies, Wilmington, DE) was interfaced to a triple quadrupole MS. Isocratic separation was achieved at 27°C on a 150 × 2.1-mm Xterra RP18 column with 3.5-µm particles (Waters Corp., Milford, MA). The mobile phase was 73% 3mM ammonium hydroxide (pH 10.5)/27% methanol delivered at a flow rate of 200 µL/min. The total analysis time per sample was approximately 5 min for a 10-µL injection.

**MS conditions.** Positive ion TurboSpray MS–MS was achieved on an API 3000 triple quadrupole MS (PE Sciex, Foster City, CA) interfaced to the aforementioned LC system. The TurboSpray source was operated at 5 kV at a temperature of 400°C and a heater gas flow of 7 L/min of nitrogen. The nebulizer gas was zero-grade air at a setting of 8 (1.04 L/min), and the curtain gas was nitrogen at a setting of 9 (1.08 L/min). The de-clustering (DP) and focusing (FP) potentials were optimized for EDEA (DP = 35 V, FP = 230 V) and MDEA (DP = 40 V, FP = 250 V). For collision-induced dissociation (CID), the collision gas was 20 eV with nitrogen as the collision gas. A collision gas setting of 11 produced a pressure reading of 3.5 to 3.7 × 10⁻⁵ Torr on the vacuum gauge. The precursor to product ion transitions used for quantitation and confirmation were measured in multiple-reaction monitoring (MRM) mode at unit resolution (Table I). The dwell time for each transition was 300 ms/cycle. No contributions from the labeled compounds to the native analytes or from the native analytes to the labeled compounds were observed.

**Data processing.** Analyst 1.1 software (PE Sciex, Foster City, CA) was used to automatically acquire and process the data. Extracted ion chromatograms were visually inspected for correct peak selection and baseline determination and were manually reintegrated as necessary. The ratio of the quantitation ion to the confirmation ion was used to detect isobaric interferences; data with ion ratios within the 99% confidence limit were accepted. Statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC) or QuattroPro9 (Corel Corp., Ottawa, ON, Canada).

**Quantitation.** Samples, blanks, standards, and QC materials were processed identically. Eight standard EDEA and MDEA concentrations (i.e., nominally 1.6, 4.0, 8.0, 25, 40, 80, 160, and 270 ng/mL) encompassing the entire linear range of the method were used to construct a calibration curve of response factor (RF, observed ratio of native peak area to labeled peak area) versus expected concentration. The eight-point calibration curve, weighted by the reciprocal of the standard concentration, was used for quantitation.

**Results and Discussion**

**Analyte extraction**

Isolation of polar, low-molecular weight compounds, such as EDEA and MDEA, from a complex and polar matrix, such as urine, was analytically challenging. SPE was employed both to remove urinary components and to facilitate enrichment of the analytes with respect to the matrix. To our knowledge, this method represents the first use of SPE in the isolation of EDEA and MDEA from urine. The method relied on the strongly basic nature (pKₐ ~9) of the analytes to effect a separation from urine using a strong cation exchange SPE column. The SPE procedure was optimized to reduce the amount of an endogenous urinary component with the same nominal mass as MDEA that was insufficiently resolved from MDEA by HPLC (vide infra).

Table II shows the means, coefficients of variation (CV), and percent recoveries for EDEA and MDEA. The recoveries of EDEA and MDEA from urine were calculated as the percentage of RFₑ/RFₑ where RFₑ and RFₑ are the response factors obtained from spiking half the samples with isotopically labeled analogues before the SPE procedure (“recovery”) and half before injection (“control”), respectively. In view of the difficulty

| Table I. Multiple Reaction Monitoring Ions for Quantitation/Confirmation of N-Ethyl Diethanolamine (EDEA) and N-Methyl Diethanolamine (MDEA) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Analyte         | Precursor Ions (m/z) | Product Ions (m/z) | Use          |
|                 | [M + H]⁺         | [M + H]⁺ &       | Use          |
| EDEA            | 134             | 134             | Quantitation |
| EDEA            | 134             | 116             | Confirmation |
| EDEA-13C₁₄     | 138             | 120             | Quantitation |
| MDEA            | 120             | 120             | Quantitation |
| MDEA-13C₁₄     | 120             | 102             | Confirmation |
| MDEA-13C₁₄     | 124             | 106             | Quantitation |

* "Recovery" denotes addition of internal standard before SPE procedure and "control" denotes addition of internal standard before injection.

| Table II. Strong Cation Exchange SPE Recovery Statistics for N-Ethyl Diethanolamine (EDEA) and N-Methyl Diethanolamine (MDEA) in Urine at Low and High Concentrations |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Analyte/Sample  | N               | Low QC Mean Conc. (ng/mL) | High QC Mean Conc. (ng/mL) | Mean CV (%) | Mean % Recovery |
|                 |                 |                 |                 |               |
| EDEA/Control    | 8               | 10.1            | 248             | 6.5          | 76             |
| MDEA/Control    | 8               | 6.96            | 209             | 17           |               |
| EDEA/Control    | 8               | 10.4            | 227             | 5.7          | 70             |
| MDEA/Control    | 8               | 7.17            | 162             | 17           |               |
in isolating low-molecular-weight polar compounds from polar matrices, in general, and the confounding factor of the interfering component, in particular, the recoveries reported here (70 to 76%) represent an acceptable compromise between recovery and clean-up. Furthermore, these recoveries compared well with the 60–80% recovery reported for the isolation of thioglycol, the analogous hydrolysis product of sulfur mustard, from urine by SPE (6). The relatively high CVs in the "recovery" samples compared with those of the "control" samples illustrate the inherent variability of the SPE process and emphasize the need for labeled reference standards to compensate for this variability.

**Chromatography and MS**

**Chromatography.** EDEA and MDEA are strongly basic aliphatic alkanolamines; these compounds displayed poor peak shapes and coeluted when acidic mobile phases were used with typical C18 columns (24). The Xterra column was chosen because of its ability to tolerate mobile-phase pH values of up to 12, allowing the analytes to be chromatographed in a neutral charge state. Ammonium hydroxide was used as a mobile-phase modifier to improve chromatography and to effect the separation of EDEA and MDEA. Analytes that can be resolved on the basis of mass-to-charge ratio usually would not require chromatographic resolution. However, in this case, two quantitation ions, [EDEA-13C4-H2O]+ and [MDEA + H]+, have the same nominal mass at m/z 120 (Δm = 0.0187), necessitating chromatographic resolution of EDEA and MDEA. Figure 1 shows a typical MRM ion chromatogram of native and labeled EDEA and MDEA from a spiked urine sample. EDEA and MDEA were well resolved (R_s > 1.5) from each other.

No urinary components were detected at the retention time for EDEA (2.99 min) in blank urine samples. In contrast, an unknown compound at 2.71 min, presumably endogenous to urine and with the same nominal mass as MDEA, was not well resolved from MDEA at 2.53 min (R_s < 1). Attempts to increase chromatographic resolution by varying the organic (methanol, acetonitrile) and aqueous mobile phase components and composition; temperature (25 to 40°C); flow rates (130 to 300 μL/min); and gradient elution were unsuccessful. Resolution was only slightly affected by changes in either temperature or flow rate; gradient elution did not result in any significant improvement over isocratic elution. Therefore, temperature was set at the lowest practical setting to conserve the column, and the flow rate was selected to minimize backpressure.

**MS.** Isotope-dilution was used to compensate for the inherent variability in urine composition, SPE methodology, and ionization efficiencies. In the product ion mass spectra of the native analytes (Figure 2) and the labeled analogues, the protonated molecular ions [M + H]+ at m/z 134 (EDEA), m/z 120 (MDEA), m/z 138 (EDEA-13C4), and m/z 124 (MDEA-13C4) were the base peaks and were, therefore, selected as the precursor ions for CID. The CID conditions for all four compounds were selected so that the precursor ion and the highest-mass product ion, represented by the neutral loss of water [M + H - 18]+ (Table I), were the two most intense ions. Maximum sensitivity was obtained by using the precursor ions for quantitation of the native analytes. For the labeled analogues, the product ions were used for quantitation because endogenous urinary compounds also exhibited protonated molecular ions at m/z 124 (MDEA-13C4) and m/z 138 (EDEA-13C4) that were insufficiently resolved from the analytes of interest by HPLC. However, CID of these endogenous protonated molecular ions did not result in the same precursor/product ion transitions that were exhibited by the labeled analogues. The presence of the product ion was used for confirmation of the native analyte, and the precursor/product ion ratio was used to verify the lack of interfering components. Ion ratios were similar for EDEA and MDEA and ranged from 1.0 to 1.5 with CVs < 10% (determined from the analysis of 20 QC materials at two concentration levels). The substantial difference between the ion ratios for the MDEA interference and MDEA is apparent in Figure 1.

The relatively low molecular weight of these analytes limited the number of sufficiently specific and intense ions available for quantitation. Thus, it was not possible to differentiate MDEA from the endogenous interference by selecting a different...
product ion because the only available ion (m/z 58) of sufficient intensity was also present in the interfering compound. However, despite incomplete chromatographic resolution, careful and consistent manual integration of MDEA produced a calibration curve comparable with that for EDEA (Table III). Although a high-resolution MS (HRMS) may be able to differentiate MDEA from the interfering compound on the basis of mass-to-charge ratio, HRMS are relatively expensive and therefore not available in most laboratories.

**Validation.** Table III shows the validation results for the calibration curves prepared in urine. For both analytes, the analysis was linear ($R = 0.9999$) over more than two orders of magnitude (from 1.6 to 270 ng/mL), and the standard errors of the estimate (expressed as CV) were < 10%. Statistical analysis indicated that for EDEA, the y-intercept was not significantly different from zero ($P = 0.4339$), confirming the absence of endogenous EDEA in the pooled blank urine used to prepare the standards. For MDEA, as expected, the y-intercept was significantly different from zero at the 99% confidence interval ($P = 0.0037$), indicating a small contribution from the interfering compound.

The method limit of detection (LOD) and limit of quantitation (LOQ) were calculated as $3s_0$ and $10s_0$, respectively (Table III), where $s_0$ is "the value of the standard deviation as the concentration approaches zero" (26). For EDEA, $s_0$ was estimated as the y-intercept of the linear regression of the standard deviation of the three lowest standards versus the expected concentration (26). The equivalent plot for MDEA resulted in a negative slope near zero; therefore, $s_0$ was calculated as the average of the standard deviations of the three lowest standards. The LOD for EDEA and MDEA were 0.41 ng/mL and 0.96 ng/mL, respectively. These values were significantly lower than values obtained by other researchers for EDEA and MDEA (i.e., 10 ng/mL) from a 20-μL injection of aqueous samples and extracts (23,24). Furthermore, the LODs reported here were comparable with those for the determination in urine of the analogous sulfur mustard hydrolysis product, thiodiglycol (1 ng/mL), by a labor-intensive method requiring derivatization followed by gas chromatography–electron capture negative-ion chemical ionization MS (6). Although no reports were found in the literature about the quantitation of EDEA or MDEA in urine after nitrogen mustard exposure, recent unpublished results from rat exposure studies in our laboratory indicated that the low LODs achieved with our method should be adequate to detect EDEA or MDEA in the urine of people exposed to nitrogen mustards.

Accuracy was evaluated in terms of the percentage of the mean calculated concentration divided by the expected concentration of spiked urine samples ($n = 5$) at each concentration level and in terms of a linear regression analysis on the plot of the calculated concentration versus the expected concentration. A slope of 1.0 indicated 100% accuracy. The accuracies were excellent (virtually 100%) for both analytes (Table III).

The day-to-day precision of the calibration standards was evaluated by calculating the CV of the calculated concentrations of spiked urine samples ($n = 5$) at each concentration level. Accuracies and precisions were averaged over the concentration range to provide mean values covering a range of 1.6 to 270 ng/mL (Table IV). For both analytes, the mean CVs were < 10%, and the mean accuracies were 100%, indicating the excellent day-to-day precision and accuracy of the method.

A standard QC chart for the concentration of MDEA in the low-level QC material is shown in Figure 3. Each point represents the single
analysis of 19 low-level QC materials analyzed over an eight-week period. CVs for all the QC materials were < 10%, reflecting excellent run-to-run variability over two months. Analysis of the QC materials one year after the initial characterization resulted in values that were within the 95% confidence intervals for both analytes at each QC concentration.

Conclusions

A simple, sensitive, rapid, accurate, and precise isotope-dilution HPLC–ESI-MS–MS method for the quantitation of EDEA and MDEA in urine at the low nanogram-per-milliliter level was developed. The method is suitable for rapid assessment of exposure to the nitrogen mustards, HN1 and HN2, respectively. Problems related to the small molecular size, polar nature, and high $pK_a$ of EDEA and MDEA made these analytes difficult to resolve from urinary components. However, despite the incomplete chromatographic resolution of MDEA from an endogenous interference, linear calibration curves and QC data for EDEA and MDEA confirmed the reliability of this method over at least two orders of magnitude.

References


