Determination of Nitrogen Mustard Hydrolysis Products in Rat Urine Samples Using GC–MS

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Abstract

A gas chromatographic-mass spectrometric method was developed, validated and demonstrated by measuring the levels of nitrogen mustard hydrolysis products in the urine collected from dosed rats. The recovery values for trimethylsilyl derivatives of EDEA and MDEA are between 82–95% and 88–112%, respectively. In vivo studies performed by using three different doses (0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg) of HN2 base of nitrogen mustard. MDEA concentrations were between 43.1–232.2 ng/mL. The limit of detection (S/N = 3) values are 2.5 ng/mL and 1.6 ng/mL for EDEA and MDEA, respectively, and the precision of the method in terms of RSD is between 5–8%.

Introduction

Nitrogen mustards, bis(2-chloroethyl)ethylamine (HN1), bis(2-chloroethyl)methylamine (HN2), and tris(2-chloroethyl)-amine (HN3) are blistering agents (1) and usage of these compounds as chemical warfare agents (CWAs) are prohibited. Altough nitrogen mustards have strong vesicant properties as sulfur mustard [2,2-di(chloroethyl)sulfide] usage of these compunds are not common because of their low volatility (2).

HN2 is primarily used clinically as an antitumor agent (3). The therapeutic use of HN2 causes significant neuronal degeneration in the absence of hypoxia when administered into bloodstream of cancer patients (4–7).

Nitrogen mustards are rapidly degraded to ethanolamines and hydrolysis products of HN1, HN2, and HN3 are ethyldiethanolamine (EDEA), methyldiethanolamine (MDEA), and triethanolamine (TEA), respectively (8). Degradation products of nitrogen mustard into ethanolamine could be used as a biomarker of incidental exposure and also it should be noted that they are far from perfect biomarkers due to their lack of specifity (9). Analysis of CWAs at trace level can be performed by coupling chromatographic techniques with spectroscopic techniques such as gas chromatography mass spectrometry (GC–MS) or liquid chromatography-mass spectrometry (LC–MS) (10–16). The prerequisite of GC–MS analysis is the analyte which has a sufficient volatility and a thermal stability. Since EDEA, MDEA, and TEA are polar compounds and their volatility is low, derivatization should be performed to convert them into non-polar compounds (17). Among derivatization reactions, conversion of the polar molecules to their silyl esters is the most common method for derivatization. The derivatization reactions of ethanolamines for GC–MS analysis involve their conversion to trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (TBDMS) esters and for this purpose derivatization reagents such as *n,o*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-methyl-*n*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) can be used. Derivatization reactions can also be used in order to improve the limit of detection values (18,19). Nitrogen mustard hydrolysis products in water and in human plasma can also be determined by GC–MS in combination with solid phase extraction (SPE) (20). Generally, the extraction step following an evaporation step has to be performed prior to derivatization and this process may lead to the loss of analytes. Ohsawa and Seto added HCl to urine matrix before evaporation and the recovery values for *tert*-butyldimethylsilyl derivatives of ethanolamines were improved (21). Dubey et al. (22) reported on-matrix derivatization-extraction (OMDEX) method for determination of nitrogen mustard hydrolysis products and improved recovery values which was obtained through a single step sample preparation technique. Other than GC–MS technique, a few studies have been reported using LC–MS for determination of ethanolamines in water and human urine samples and the major advantage of LC–MS is that it avoids derivatization during sample preparation (23,24).

The purpose of this study was to develop an analytical method to determine the ethanolamine concentrations in urine samples of rats which were exposed to mechlorethamine (HN2 base) at different doses using GC–MS.

Experimental

Materials

n-Ethyldiethanolamine (EDEA) and *n*-methyldiethanolamine (MDEA) stock solutions, HN2 base of nitrogen mustard Mechlorethamine HCl, derivatization reagent *n,o*-bis(trimethylsilyl)-trifluoracetamide [BSTFA with 1% trimethylchlorosilane (TMCS)] and nonadecane, as internal standard (IS), were obtained from Sigma-Aldrich (Ankara, Turkey). Hydrochloric

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acid (HCl), methanol, and acetonitrile were obtained from Merck (Ankara, Turkey).

All of the stock and working standard solutions of chemicals were prepared daily in acetonitrile. EDEA and MDEA calibration standards were prepared by appropriate dilutions of a stock solution of 10 µg/mL which contains both analytes. The IS stock solution at a concentration of 1000 µg/mL was prepared by dissolving appropriate amount of nonadecane in acetonitrile. Working standard solution of IS at a concentration of 10 µg/mL was used throughout the experiment. All of the reagents were analytical grade. Mechlorethamine was prepared in physiological saline at a concentration of 200 mg/L for intraperitoneal injection (intraperitoneal injection is the injection of a substance into the body cavity) to the male Sprague–Dawley rats (170–200 g).

Instrumentation

GC–MS system consisted of an Agilent 7890 gas chromatograph interfaced with an Agilent 5975 C quadropole mass spectrometer (Agilent Technologies, Santa Clara, CA) was used throughout the experiment. DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm thickness, J&W Scientific, Folsom, CA) was used for chromatographic separations. Helium (He) was used as carrier gas at a constant flow rate of 1 mL/min. Split injection mode was used with the split ratio of 10:1. Injection port and transfer line temperatures were set to 250°C and 280°C respectively. The temperature program used for separation of the analytes was: the initial temperature 90°C (2 min hold), then ramp to 290°C at 15°C/min (5 min hold). Mass acquisition was started 4 min after sample injection. Electron ionization was used with 70 eV ionization energy for the experiment. The ion source and quadrupole mass analyzer temperatures were maintained at 230°C and 150°C, respectively.

A nitrogen evaporation system which was used for evaporating urine samples was constructed in our laboratory to evaporate the urine samples.

The determination of EDEA and MDEA was performed using selected ion monitoring (SIM) mode. The retention times and selected ions to monitor trimethylsilyl (TMS) derivatives of EDEA, MDEA, and IS were given in Table I. The ions given in Table I were monitored in order to obtain sufficient certainty and the quantification was performed by using the *m/z* values 160, 174, 268 for MDEA, EDEA, and IS, respectively.

Intraperitoneal application of Mechlorethamine (HN2)

The animals were housed in stainless steel cages in a controlled environment (20°C, 50% relative humidity and 12 h light/12 h dark cycle) and allowed free access to standard rat chow and tap water. Animal experiments were carried out in accordance with the Guidelines for Animal Experimental of Gulhane Military Medical Academy (Ankara, Turkey), and the protocol was approved by the Animal Ethics Committee.

Intraperitoneal application of HN2 was done in three sets of dosing 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and a control group and each group consists of 7 rats. Before the application, the rats were weighed and the HN2 injection volume was calculated according to their weights. The collection of rat urine samples was carried out in metabolism cages and after 24 h from dosing HN2 administration urine samples were collected and analyzed with the proposed method. For the control group physiological saline was injected.

Table I. The Retention Time and Ions Selected to Monitor Trimethylsilyl (TMS) Derivatives of MDEA, EDEA, and IS

	Retention time (min)	Ions monitored (relative abundance)
MDEA	6.6	160 (100), 73 (47)
EDEA	7.1	174 (100), 73 (51)
Internal standard	11.4	57 (100), 268 (22)

Sample preparation

It is known that adding HCl before the evaporation step improves the recovery values of ethanonamine derivatives (21) therefore, the final HCl concentration was optimized in the range of 0.01–0.2 M and the results were shown in Figure 1. Since the recovery values for both analyte were too low up to 0.05 M, before the evaporation step final HCl concentration 0.1 M was used throughout the experiments.

For sample preparation, 100 µL aliquot of rat urine sample was placed in a micro centrifuge tube and 400 µL methanol was added for deproteinization. Then, the sample was centrifuged at 4000 g for 10 minutes. Supernatant was taken in a glass vial and 20 µL of 3.0 M HCl was added to avoid the loss of ethanolamines during evaporation and the sample was evaporated to dryness at 40°C under gentle stream of nitrogen. Every sample was prepared in five replicate. Using single step sample preparation simplified the analysis procedure and could save some time for sample preparation, especially with biological samples.

Derivatization

In order to check the applicability of the method, the metabolites were derivatized to their corresponding trimethylsilyl derivatives (Figure 2). Evaporated urine samples were dissolved with 50 µL of acetonitrile containing 0.25 µg IS. The samples were vortexed for one min. Then, 50 µL BSTFA was added and incubated at 60°C for 30 min.

In order to prepare the calibration standards, 25 µL of standard solution containing appropriate amounts of ethanolamines (final concentrations were between 5–800 ng/mL) and 25 µL IS at a concentration of 10 µg/mL (0.25 µg) was added in a glass

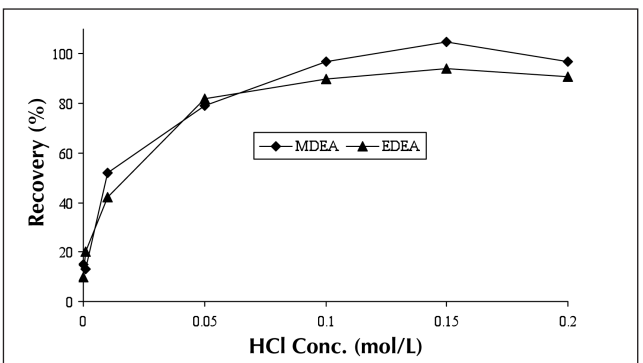

Figure 1. Effect of HCl concentration on the recovery of ethanolamines from spiked urine samples (EDEA and MDEA concentration is 100 ng/mL).

vial. After vortexing for 1 min, 50 µL BSTFA was added and incubated at 60°C for 30 min. One microliter of this solution was injected to GC–MS system. Total ion chromatogram obtained for the spiked urine sample in Scan mode for IS and TMS derivatives of MDEA and EDEA was shown in Figure 3.

In order to investigate the derivative stability, the urine samples were treated as mentioned above and the samples were stored at 4°C. GC–MS analysis was performed everyday for 4 days. The results indicated that the derivatives of EDEA and MDEA could be stored at 4°C for two days without any significant degradation.

Results and Discussion

Method validation

Although only HN2 base was used in animal experiments and hydrolysis product MDEA was determined, the validation of the method was performed with both hydrolysis products of HN1 and HN2 bases in order to point out that both of the hydrolysis products (EDEA and MDEA) could be determined accurately at the same time. Therefore, urine samples of control group were collected and spiked with four different concentrations (10 ng/mL, 25 ng/mL, 50 ng/mL, and 250 ng/mL) of EDEA and MDEA in order to validate the proposed method and every sample was prepared in five replicate. The spiked samples were treated same described previously. The recovery values for EDEA and MDEA were between 82–95% and 88–112%, respectively (Table II).

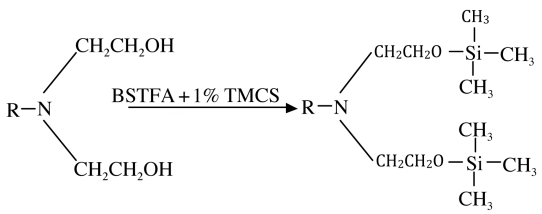

Figure 2. Derivatization reaction of MDEA and EDEA, where R is CH_3 and C_2H_5 for MDEA and EDEA, respectively.

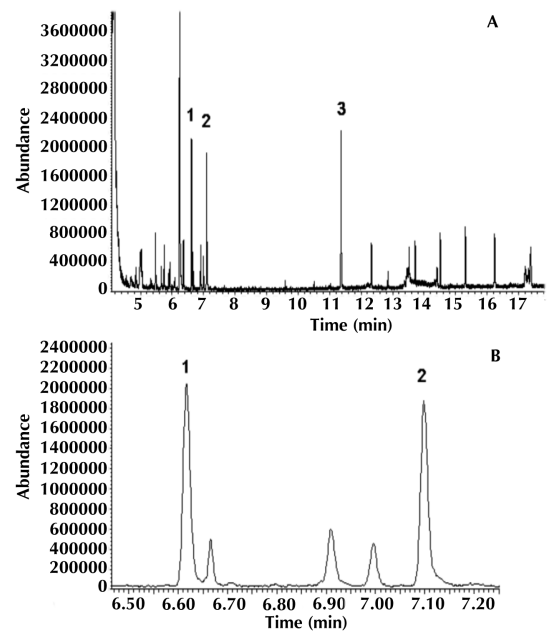

Figure 3. (A) Total ion chromatogram (TIC) obtained in scan mode for spiked urine sample. The concentrations of IS and TMS derivatives of ethanolamines are 2.5 µg/mL and 0.5 µg/mL, respectively: (1) MDEA, (2) EDEA, (3) IS. (B) The enlarged view of TIC.

MDEA determination in rat urine samples

Nitrogen mustard as a vesicant agent is unstable compound in both environmental and biological medium and degrades to yield its corresponding metabolites. The detection of the metabolites in biological samples provides a possible indication of the presence of mustard. In terms of simulating a chemical incident, HN2 base of nitrogen mustard was applied intraperitoneally to the rats and hydrolysis product of nitrogen mustard, MDEA, was determined. Seven rats were used for each dose group and 5 replicates were prepared using the urine collected from each rat. The MDEA concentrations corresponding to applied doses of HN2 are shown in Table III. MDEA concentrations differ between 43.1–60.1 ng/mL, 88.1–138.4 ng/mL, and 178.1–232.2 ng/mL for the doses 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg, respectively. In order to investigate the potential interferences, the collected urine samples of control group were used as sample blank and any signal resulting from sample blank was not observed during the experiments. Also the results indicate that the LOD achieved with this method is adequate for quantitation of nitrogen mustard after an exposure. Lemire et al. (25) determined nitrogen mustard hydrolysis products in rat urine samples using LC–MS after dermal application and rat dosing studies confirmed unconjugated ethanolamaines.

Analytical figures of merit

The analytical figures of merit of the proposed method for the determination of EDEA and MDEA in urine samples are shown in Table IV. The linearity of the method in SIM mode was ensured by using a series of standard ethanolamine solutions at concentrations 5–800 ng/mL and each of the calibration standards contains IS at a final concentration of 2.5 µg/mL. The calibration curves were constructed using 5 calibration standards and the ratio of peak areas of analyte to that of internal standard was measured. Five replicate measurements were performed for each sample. The least squares regression analyses of the calibration curve for EDEA is 0.1185[EDEA] + 0.4023 (r^2 = 0.9921) and for MDEA is

Table II. Determination of EDEA and MDEA in Spiked Urine Samples*

EDEA and MDEA added (ng/mL)	EDEA recovered (%)	MDEA recovered (%)
10 (EDEA) + 10 (MDEA)	82 ± 7.2	88 ± 9.1
25 (EDEA) + 25 (MDEA)	86 ± 4.4	94 ± 5.8
50 (EDEA) + 50 (MDEA)	95 ± 2.7	112 ± 7.4
250 (EDEA) + 250 (MDEA)	92 ± 5.8	106 ± 6.1

*Results are given as average ± standard deviation (n = 5).

Table III. Conc. of MDEA Determined in Urine Samples of Rats Dosed with HN2*

Dose (mg/kg)	Concentration (ng/mL)						
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
0.5	53.1 ± 2.2	56.8 ± 4.3	51.6 ± 3.8	43.1 ± 4.8	59.3 ± 3.7	45.1 ± 4.6	60.1 ± 4.6
1.0	102.3 ± 4.1	114.7 ± 3.8	138.4 ± 4.9	97.2 ± 5.6	111.4 ± 5.6	88.1 ± 2.8	117.2 ± 3.1
2.0	185.7 ± 4.4	213.4 ± 8.6	232.2 ± 9.2	195.6 ± 8.6	178.1 ± 3.1	210.1 ± 6.8	202.5 ± 7.7

*Results are given as average ± standard deviation (n = 5)

Table IV. Analytical Figures of Merit of the Proposed Method for Determination of EDEA and MDEA using GC–MS

Parameter	EDEA	MDEA
Linear range, ng/mL	5–800	5–800
Relative standard deviation (n = 9), %	7.2	5.8
Limit of detection (LOD), ng/mL	2.5	1.6

0.1112[MDEA] + 0.1045 (r^2 = 0.9983). The linear range of the method was 5–800 ng/mL for both analytes. The precision of the method for determination of EDEA and MDEA in terms of relative standard deviation (RSD) was between 5–8%.

The limit of detection (LOD) values for EDEA and MDEA were calculated according to the concentration required for giving a peak height three times higher than background noise (S/N = 3). LOD values for EDEA and MDEA were calculated as 2.5 ng/mL and 1.6 ng/mL, respectively by using a standard at a concentration of 5 ng/mL. The obtained LOD values were comparable with the values obtained with trifluoroacetyl derivatives (26).

Conclusion

The HN2 form of nitrogen mustard has been used as a chemotherapeutic agent but on the other hand it is also known as one of the CWAs, so after an exposure, the concentration of nitrogen mustard has to be determined in order to carry out a convenient clinical approach. In this study, GC–MS method is demonstrated for the determination of nitrogen mustard hydrolysis products and the method was successfully run in the urine samples. The demonstration of these products in urine has been found quite useful biological marker for the unequivocal verification of mustard exposure. Since the demonstrated method does not involve an extraction step, it is relatively less time consuming. The obtained analytical figures of merit are comparable with the other methods in the literature and can also be used as an alternative to existing methods.

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