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High-throughput sample preparation and simultaneous column regeneration liquid chromatography–tandem mass spectrometry method for determination of nitrogen mustard metabolites in human urine

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ABSTRACT

Nitrogen mustards (NMs) are known to have DNA alkylation and strong vesicant properties. Their availability to terrorist organizations makes them a potential choice for chemical attacks on civilian populations. After an exposure, it is difficult to measure NMs directly because of their rapid metabolism in the human body. Therefore to determine an individual's level of exposure to NMs, it is necessary to analyze for NM metabolites being excreted by the body. The metabolites of NMs are generated by a hydrolysis reaction, and are easily detectable by liquid chromatography tandem mass spectrometry (LC–MS/MS). This work is focused on the development of a high-throughput assay for the quantitation of N-ethyldiethanolamine (EDEA) and N-methyldiethanolamine (MDEA) metabolites of bis (2-chloroethyl) ethylethanamine (HN1) and bis (2-chloroethyl) methylethanamine (HN2), respectively. The method uses automated 96-well plate sample preparation of human urine samples and a 2-position 10-port switching valve to allow for simultaneous regeneration of the liquid chromatography (LC) columns. Using this method, over 18 h was saved through the reduction of sample preparation and analysis time when compared to a conventional method for 96 samples. The validated method provided excellent accuracy for both EDEA (100.9%) and MDEA (100.6%) with precision better than 5.27% for each analyte.

1. Introduction

The deliberate use of chemicals in terrorist activities is one of the greatest potential threats to human beings across the globe. Nitrogen mustards (NMs) are blister causing agents, which attack the central nervous system [1]. They are also bifunctional alkylating agents that may covalently bond to DNA and proteins, causing cytotoxicity [2–5]. The NMs, bis (2-chloroethyl) ethylethanamine (HN1), bis (2-chloroethyl) methylethanamine (HN2), and tris (2-chloroethyl) amine (HN3) are listed on the Chemical Weapons Convention Schedule of Chemicals [6].

The high reactivity and short lifetime of these compounds makes it difficult to directly measure NMs in exposed individuals. Hence the hydrolyzed metabolites of NMs are better biomarkers for assessing exposure to HN1, HN2, and HN3 [7]. However, as previously reported, the HN3 hydrolysis product N-triethanolamine (TEA) is a common ingredient in a wide variety of consumer products [8]. The prevalence of this compound in products like cosmetics and other personal care items has resulted in a significant amount of TEA being detectable in the background population. Studies to evaluate its background level have found that as many as 47% of individuals tested for TEA show elevated levels [9]. To this end, TEA is not a reliable biomarker of HN3, and was not evaluated in this assay.

In previously reported methods for the detection of EDEA and MDEA in environmental samples, several gas chromatography–mass spectrometry (GC–MS) protocols were presented [10–13]. As part of the Chemical Weapons Convention, Kanaujia et al. performed a comparative SPE extraction using strong cation and mixed mode strong cation-exchange cartridges followed by GC–MS analysis of amino alcohols in water and human plasma [14]. The high polarity, basic nature, and low volatility of these analytes required derivatization and extensive sample preparation, as described by Black and Muir [15]. These prolonged sample preparation steps limit the application of GC–MS methods in the analysis of amino alcohols.

The LC–MS analysis of several NM degradation products, including EDEA and MDEA, in water and decontamination solutions has been reported [16]. The determination of ethanolamines in high salinity industrial wastewater was also reported using LC and tandem mass spectrometry [17]. Atmospheric pressure chemical ionization (APCI) in combination with liquid chromatog-

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raphy tandem mass spectrometry (LC–APCI-MS/MS) methods have advantages over GC/MS methods in the quantitation of chemical warfare agents because of simple sample preparation steps and easy analysis procedure [18]. Finally, nitrogen mustard adducted with DNA, and depurination products such as the N-7-alkyl guanines (N-7-G) in urine, can also be used as biomarkers for the determination of NM's [5,19]. Still, each reported method has its limitations and none of the previously reported methods have ever been applied to determine EDEA and MDEA urinary metabolites after exposure to NMs [9].

A solid phase extraction (SPE), liquid chromatography electrospray–tandem mass spectrometry (LC–ESI-MS/MS) method using isotopically labeled internal standards of EDEA and MDEA in biological matrices was published by Lemire et al. [20]. Subsequently, further modifications were made by this same group to optimize the chromatographic separation of EDEA and MDEA [9]. A survey of the literature indicates that there is still great need for a robust and high-throughput method to detect NMs after a large-scale exposure event.

The present study describes an automated, high-throughput extraction, with alternating LC column regeneration by valve switching. A 2-position 10-port valve is utilized to switch flows between columns, allowing for regeneration of one column while separation is performed on the other. The detection and quantitation of EDEA and MDEA (Fig. 1) in urine is achieved using LC–ESI-MS/MS. In addition to higher analytical throughput, this method uses less sample volume (300 μL), requiring fewer resources and labor, without sacrificing precision and/or accuracy.

2. Experimental

2.1. Chemicals and reagents

A mixture of EDEA and MDEA standards in urine were obtained from SPEX CertiPrep Group (Metuchen, NJ, USA) in concentrations of 1, 5, 10, 50, 100, 250, and 500 ng/mL in sealed ampoules. A certified urine blank (1.8 mL), and two quality-control standards (QC) were also provided. The nominal concentrations of the QC low and high materials were 25 and 300 ng/mL, respectively. An isotopically labeled internal standard solution of EDEA-$^{13}C_4$ and MDEA-$^{13}C_4$ at a concentration of 1000 ng/mL in 15 mM ammonium hydroxide solution was also obtained from SPEX CertiPrep in 1.8 mL flame sealed ampoules. Extraction was performed using Varian Bond Elut Certify (100 mg/well) solid phase extraction (SPE) 96-well plates (Lake Forest, CA, USA). Two Luna CN columns (100 mm × 2 mm, 3 μm) from Phenomenex (Torrance, CA, USA) were used as column A and column B for liquid chromatography analysis. Strata 96 square well (2 mL/well) polypropylene plates (Phenomenex) were used for initial sample aliquots and collection after extractions. HPLC grade methanol, HPLC grade acetonitrile, ammonium hydroxide solution, and ammonium bicarbonate were obtained from ThermoFisher Scientific (Pittsburgh, PA, USA). Organic-free 18.3 MΩ cm^{-1} ultra pure water was purified in-house using an E-pure system (Barnstead International, Dubuque, IA, USA). Pooled urine was donated by healthy laboratory personnel.

2.2. Instrumentation

A Tecan Freedom EVO® 200 (Research Triangle Park, NC, USA) liquid handling system equipped with an eight-channel liquid handling arm (LiHa) for pipetting tasks was used for high throughput extraction and sample preparation. The system also included a mounted Te-VacS plate vacuum manifold, Te-Shake plate vortexer, and robotic manipulator arm (RoMa) with gripper fingers for transporting plates around the work deck. Following SPE, sample extracts were concentrated to dryness using a TurboVap® 96 evaporator (Caliper Life Sciences, Hopkinton, MA, USA) under N_2. For comparison a Gilson 215 Liquid Handler (Middleton, WI, USA) was used for barrel SPE extraction and sample preparation. The sample extracts were concentrated to dryness under N_2 using a TurboVap® LV (Caliper Life Sciences). LC column switching was performed using a 2-position 10-port Valco valve (VICI® Valco Instruments Co. Inc., Houston, TX, USA). Liquid chromatography was performed using an Agilent 1200 HPLC system (Agilent Technologies, Wilmington, DE, USA). LC column regeneration was accomplished using an Agilent 1100 isocratic pump. Mass spectrometry analysis was performed with a 4000 QTRAP™ mass spectrometer (AB Sciex, Foster City, CA, USA). The LC–MS/MS system configuration, sample analysis, and data collection were performed using Analyst™ software version 1.4.2 (AB Sciex).

2.3. 96-well plate extraction and sample preparation

Three-hundred microliters of each calibration standard and QC standard was manually transferred into a clean 96-well plate. The 96-well plate with sample aliquots was placed in the Tecan for automated sample extraction. The LiHa arm was used to add 20 μL of internal standard to each sample well. Each sample was diluted with 300 μL of organic-free ultrapure H_2O, and vortex mixed for 35 s by the Te-shake at 1500 rpm. Each well of the SPE plate was conditioned with 1.0 mL of MeOH followed by vacuum. The plate was then equilibrated with 1.0 mL of 18.3 MΩ H_2O, and vacuum was applied. The LiHa arm was then used to transfer the total volume (620 μL) of prepared sample to the SPE plate followed by vacuum. The SPE beds were then washed with 1.0 mL of acetonitrile under minimal vacuum to remove residual water from the stationary phase. Excess water present in the sample can result in inconsistent sample evaporation. Sample elution into a clean 96-well collection plate was performed with 2 cycles of 750 μL of 10% NH_4OH in acetonitrile (v/v), each with the application of vacuum. The elution plate was evaporated to dryness using a TurboVap 96 under N_2 at 40 °C. Finally, each sample was reconstituted with 100 μL of 10 mM NH_4HCO_3 solution using the Tecan Freedom EVO. The 96-well plate extraction was accomplished using lower sample volume (300 μL) with shorter sample preparation time. The automated system eliminated a number of manual transfer steps, thus minimizing errors, and the time required for extraction of 96 samples was 45 min.

2.3.1. Gilson sample preparation

For comparison, a Gilson 215 Liquid Handler extraction was performed employing a 3cc Varian Bond Elut Certify Tabless SPE cartridge (300 mg bed). A 1 mL sample aliquot was used for extraction, and the extract was concentrated to dryness under N_2 at 40 °C using a TurboVap. The sample was reconstituted into 150 μL of 10 mM NH_4HCO_3 and transferred into 1.5 mL sample vials with inserts.

2.4. Validation

2.4.1. Linearity

The linearity of the method was determined by analysis of standard plots associated with a seven-point standard calibration curve within the range 1–500 ng/mL. The calculation was based on the peak area ratio of analyte versus the area of internal standard. Linearity was observed over the specified concentration range with $r = 0.9997$ and 0.9998 for MDEA and EDEA, respectively.

2.4.2. Limit of quantitation (LOQ)

The lowest concentration of calibration standard of MDEA and EDEA, 30 pg on-column, represented the limit of quantitation (LOQ)

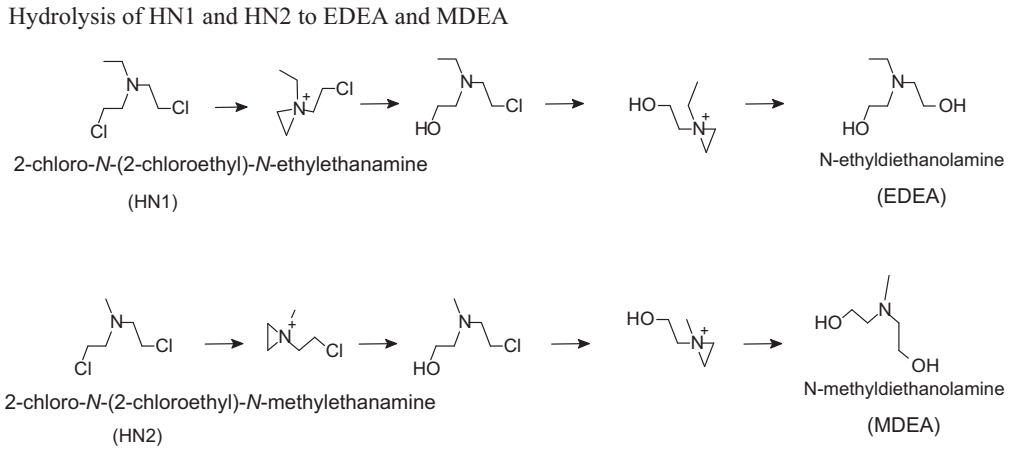

Fig. 1. Hydrolysis of HN1 and HN2 to EDEA and MDEA.

Table 1
Precision and accuracy of the method.

	MDEA					EDEA				
	Intra-day		Inter-day			Intra-day		Inter-day		
	QL	QH	QL	QH	Accuracy	QL	QH	QL	QH	Accuracy
	24.7	300	24.2	311	96.7	25.6	301	24.9	305	102
	26.9	320	25.0	299	100	27.7	302	24.9	304	101
	24.9	295	23.3	302	93.1	26.2	305	24.9	298	99.4
	25.5	301	25.9	296	104	25.8	303	26.3	299	99.7
	24.9	301	25.9	310	104	26.0	302	26.7	318	106
	25.3	302	26.4	294	106	24.3	302	26.1	291	97.1
Mean	25.3	303.2	25.1	302	100.6	25.9	302.5	25.6	302.5	100.9
%RSD	3.21	2.84	5.27	2.36		4.72	0.46	3.22	3.01	

Note: QL = quality control low concentration; QH = quality control high concentration.

of the assay. The LOQ for both of the analytes were determined based on the signal to noise ratio (S/N) ≥ 10 for the lowest calibrator.

2.4.3. Matrix interference

The total ion chromatograms of certified urine blank Fig. 4(a) and spiked urine with MDEA and EDEA internal standards Fig. 4(b) were compared to determine the interferences of endogenous matrix components. No interfering endogenous peaks were found in the retention time range of the target analytes.

2.4.4. Accuracy and precision

Intra-day and inter-day precision, and accuracy were measured using two levels of quality-control samples for both MDEA and EDEA ($n = 6$) in urine. The levels of quality-control injected were 750 pg and 9000 pg on column. Intra- and inter-day precision was determined by calculating the % relative standard deviation (%RSD) from six different measurements ($n = 6$) of each analyte at each QC level. The accuracy was calculated as the percentage ratio between measured and theoretical concentrations. Intra-day and inter-day precision, and accuracy of QC-low and QC-high for the both analytes were very consistent as shown in Table 1.

3. Results and discussion

3.1. Development of column switching – Valco valve method

A time saving simultaneous LC column regeneration method was developed using an external 2-position 10-port Valco valve, the valve performed simultaneous LC column switching between column A and B. All ports in the valve were connected as shown in Fig. 2, and externally connected to an Agilent 1200 HPLC thermostated column compartment. Two separate methods, one for column A and another for column B were entered into the Analyst software. When creating the sample batch file, the 'use multiple methods' option was selected, and methods A and B were alternated for each sample in the batch. Thus, valve switching allowed method A to utilize column A and method B to use column B. The valve was configured in diverter mode and the mass spectrometer was configured in LC-synchronization mode. The timing and positions of the valve for LC method A and method B are shown in Table 2.

3.2. Automated 96-well plate SPE vs. automated cartridge SPE

A robust 96-well plate sample preparation method was developed using Tecan EVOware. The robustness of the 96-well plate extraction method was compared to the results obtained from samples extracted using a Gilson 215 SPE method. All of the samples extracted with both methods were analyzed with the same column switching method, and under identical mass spectrometry

Table 2
Valco valve switching times in method A and B for LC column A and B.

Method A		Method B	
Total time (min)	Valve position	Total time (min)	Valve position
0.1	A	0.1	B
0.5	A	0.5	B
1.0	A	1.0	B
3.2	A	3.2	B
5.5	A	5.5	B
5.6	B	5.6	A

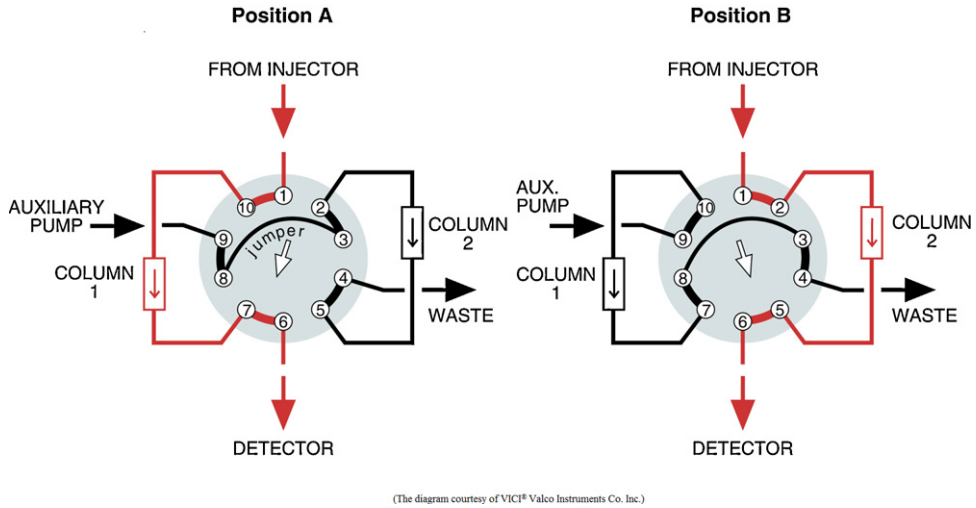

Fig. 2. Configuration of Valco valve A and B. Position A directs column flow to mass spectrometer through column 1 (A) while column 2 (B) is regenerated. Position B reverses the process.

conditions. The time taken to extract 96 samples in both methods was compared. The total sample preparation time using the 96-well plate method was 45 min, compared to 288 min for the Gilson cartridge method. Additionally, the cartridge sample preparation method required 1.0 mL of sample and more solvent, while the 96-well plate sample preparation method required only 300 μL of sample. Extracted ion chromatograms obtained for samples from each sample preparation procedure have shown consistency (Fig. 3), even with lower volumes of MDEA and EDEA calibrators in Tecan sample preparation method (30–15,000 pg on column) compared to Gilson sample preparation method (60–33,300 pg on column).

3.3. Chromatography

The new LC conditions were developed to achieve the optimal chromatographic separation of MDEA and EDEA, while significantly reducing the retention times of the target analytes. As shown in Fig. 4(b), the MDEA and EDEA had retention times of 2.67 min and 3.18 min, respectively. Chromatographic separation was achieved using a 10:90 binary mobile phase of 10 mM

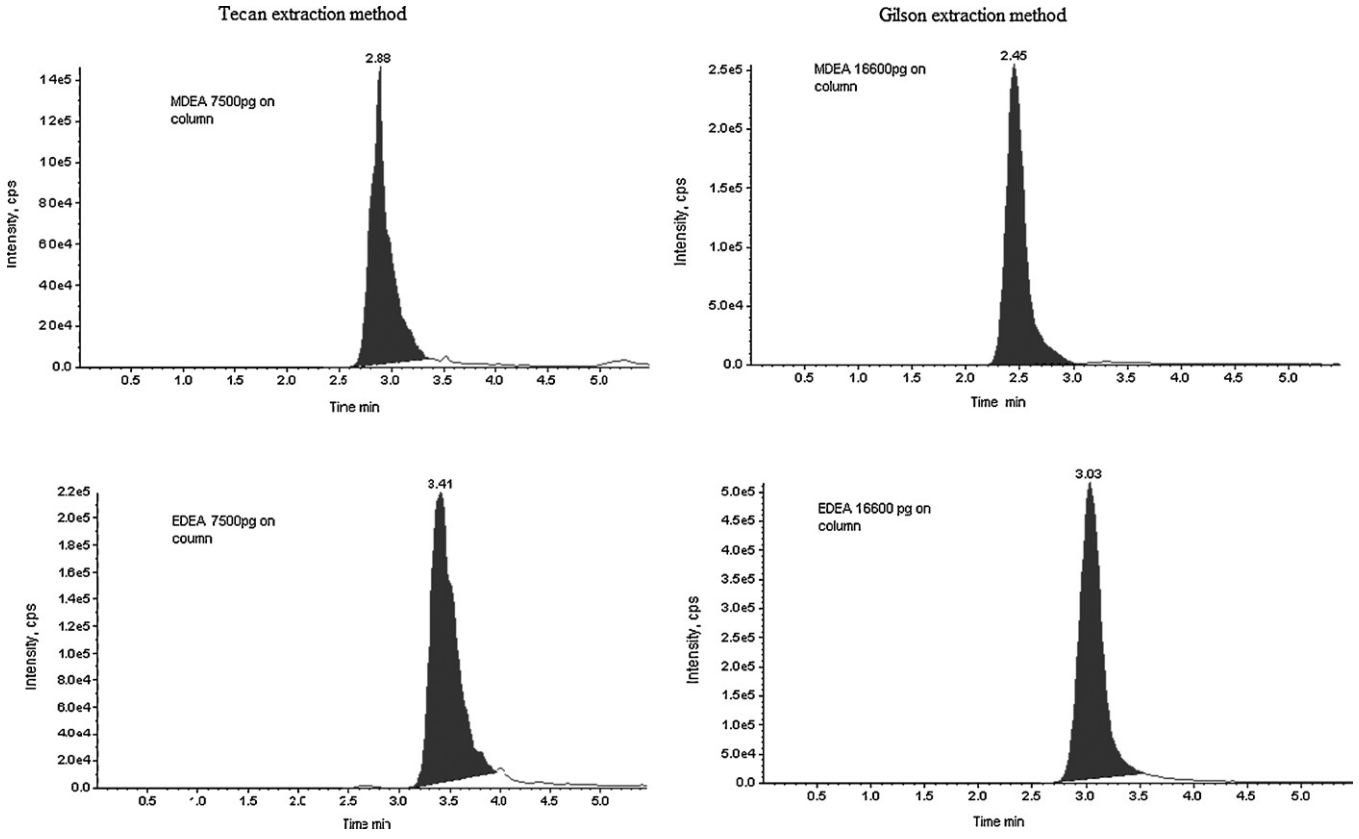

Fig. 3. Extracted ion chromatograms of MDEA and EDEA.

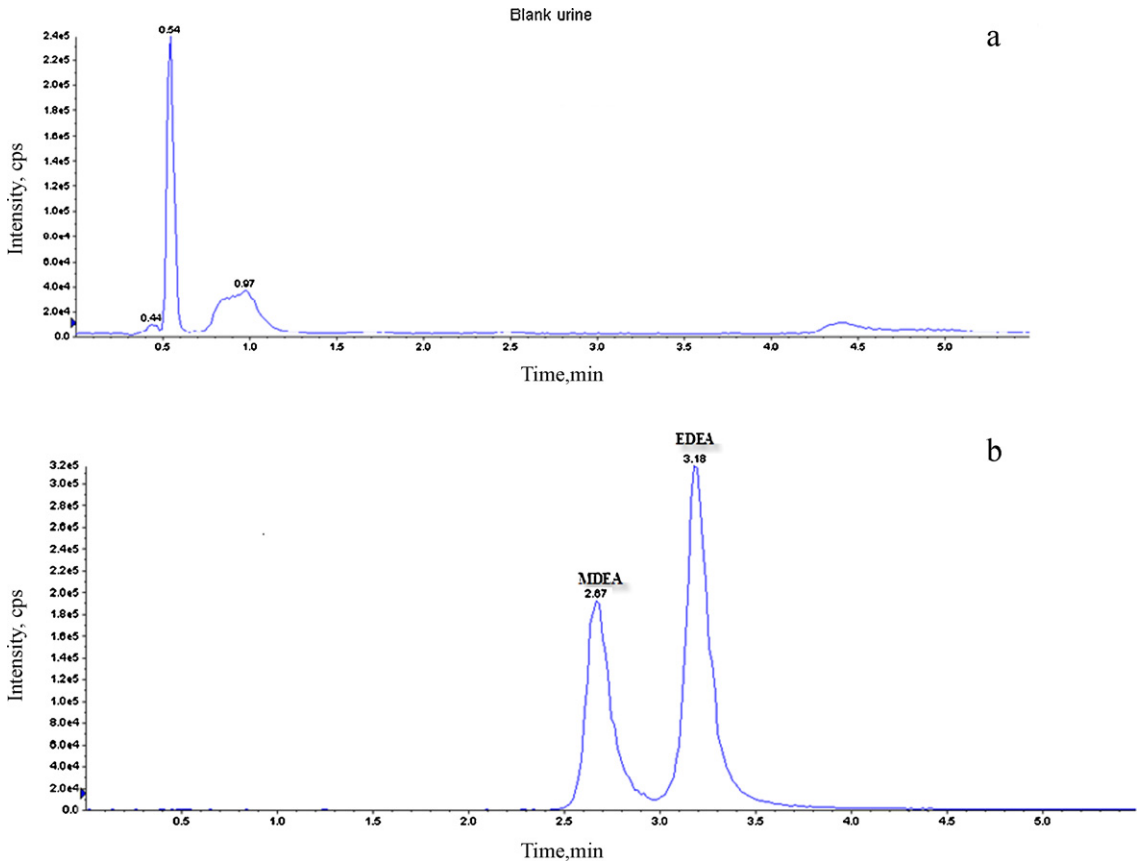

Fig. 4. Total ion chromatogram for (a) certified urine blank, and (b) spiked internal standards of MDEA and EDEA.

NH_4HCO_3 and Methanol at a flow rate of 0.5 mL/min, on two Luna CN columns. Clean up and re-equilibration of the LC columns was performed with the same mobile phase, at a flow rate of 0.5 mL/min. With each 10 μL sample injection, one column was used for chromatographic separation while the other column was being regenerated using the isocratic pump by switching the valve between position A and position B at 5.6 min intervals. Both analytes were eluted with consistent retention times and proper peak shape. Under the basic pH conditions used, MDEA and EDEA remain in their charged cationic state [14], and were retained without difficulty. The new method was rapid, with a total of 10.9 min per run saved. The analysis of 96 samples using a conventional single column method required 1440 min, while column switching method required 528 min.

3.4. Mass spectrometry detection

Positive electrospray ionization, tandem mass spectrometry experiments were performed for each analyte using multiple-reaction monitoring (MRM). Two ion transitions for each target analyte were selected for monitoring, one transition was used for quantitation and the other for confirmation. The following ion transitions were used: m/z 120.1 → 102.1, m/z 120.1 → 58.1, and m/z 124.1 → 106.1 for quantitation, confirmation, and internal standard of MDEA, and m/z 134.1 → 116.1, m/z 134.1 → 72.1, and m/z 138.1 → 120.1 for quantitation, confirmation, and internal standard of EDEA, respectively. The collision energy (CE), de-clustering potential (DP), collusion cell exit potential (CXP), and entrance potential (EP) for specified transitions of MDEA and EDEA were optimized. The collision induced dissociation (CID) spectra were recorded using nitrogen as collision gas. The Turbolon spray source was operated at 5000 V and temperature at 300 °C. The collision gas, gas 1 and gas 2 were used with optimized setting of 9, 20, and 20, respectively for MDEA and EDEA.

4. Application of the method to unknown samples

For the further evaluation of the applicability of the method, the proposed LC-column switch and high through sample preparation method was successfully applied to the analysis of unknown and QC urine samples in the quantitative study of MDEA and EDEA. Both the analyte concentrations in a set of QCs and unknown spiked samples were determined using calibration curves. The results are shown in Table 3, and proved the rapid and robustness of the method.

Table 3
Data of unknown spiked samples and QCs of MDEA and EDEA.

Sample	MDEA		EDEA	
	Spiked Quan.	Measured Quan.	Spiked Quan.	Measured Quan.
1	0.0	0.0	20.9	21.1
2	0.0	0.0	8.8	8.8
3	55.0	53.3	57.0	56.8
4	7.6	7.1	0.0	0.0
5	30.7	31.3	199.0	194.0
6	0.0	0.0	388.0	403.0
7	81.1	79.4	122.0	116.0
8	291.0	288.0	3.6	3.7
9	175	174.0	175.0	171.0
10	20.2	19.1	0.0	0.0
QC	25.0	24.0	25.0	24.2
QC	300.0	298.0	300.0	301.0

Note: Spiked and measured quantities of samples are expressed in ng/mL.

5. Conclusions

The combination of automated 96-well plate SPE techniques and LC column switching offered an 18.4 h time savings for a batch of 96 samples by drastically increasing sample throughput when compared to previously reported methods. These improvements conserved sample resources and minimized the risk of manual errors while maintaining sufficient sensitivity. These modifications will enable responding laboratories to better handle large numbers of samples that are projected to be associated with a large scale nitrogen mustard exposure event.

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