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Quantitative Determination of Tetramethylammonium in Blood and Urine Samples by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Tetramethylammonium (tetramine), a highly toxic compound, is found in high levels in the salivary glands of the sea snail, Neptunea. Tetramine neurotoxicity is fatal, and therefore, prompt determination of tetramine in patients' biological samples is important in guiding proper and timely treatment. However, there are only a few reports on the quantitative determination of tetramine in human's biological samples. In the present study, we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with simple protein precipitation for sample extraction. Using the method, we performed a quantitative determination of tetramine in blood and urine samples of a couple who were transferred to a hospital emergency room mentioned after consuming Neptunea. The validation parameters including linearity, accuracy, precision, matrix effect, and recovery were satisfactory. The concentrations of tetramine in the husband's femoral vein blood and urine were 1.37 mg/L and 15.07 mg/L, respectively, and 0.57 mg/L and 5.85 mg/L, in the wife's blood and urine, respectively. Since few studies have reported toxic and fatal levels of tetramine in blood, this study can be a reference to evaluate tetramine poisoning in the clinical and postmortem toxicology.

Keywords: Tetramethylammonium (Tetramine); LC-MS/MS; Blood; Urine; Hospital emergency room

Introduction

Tetramine is a highly toxic compound, found in high levels in the salivary glands of some gastropods such as Neptunea arthritica and Neptunea intersculpta [1]. Which are commonly found at the South Korean coast. It is known to be a potent neurotoxin owing to its chemical structure. The chemical structure of tetramine contains quaternary ammonium, and is similar to acetylcholine. The neurotoxicity of tetramine may be due to binding and activating nicotinic-acetylcholine receptors causing difficulties in breathing, muscular paralysis, and possible death [1]. Other symptoms of tetramine poisoning include dizziness, blurred vision, bilateral leg weakness, and gait disturbance [2]. In South Korea, several cases of tetramine poisoning associated with the consumption of Neptunea, have been reported [2-5]. Tetramine neurotoxicity is fatal, and therefore, prompt determination of tetramine in patients' biological samples is important in guiding proper and timely treatment. However, there are only a few reports on the quantitative determination of tetramine in the patients' biological samples. Tetramine has low molecular weight and high polarity, therefore, Gas Chromatography/Mass Spectrometry (GC/MS) is not an efficient method for quantitative analysis of tetramine in biological samples including blood and urine. On the other hand, a previous study reported successful use of ion chromatography and LC-MS/MS for the quantitative analysis of tetramine in Neptunea collected from South Korea's coastal regions [6]. Based on these findings, we hypothesized that an LC-MS/MS method would be efficient in quantitative analysis of tetramine in human' biological samples. In this study, we developed an LC-MS/MS method and a protein precipitation method for quantitative analysis of tetramine in blood and urine, and used them to analyze two patients' biological samples.

Case Report

A couple, a 67-year-old male and a 65-year-old female were transferred to a hospital emergency room on November 1st, 2020 complaining of stomachache after eating several sea snails. They were in a mental stupor and semi-coma state. All the laboratory results were within the normal range. After the patient gave informed consent, the blood and urine samples were sent to the Busan Institute of National Forensic Service for toxicological examination which has been designated as regional poisoning analysis laboratory from Korean National Medical Center.

Materials and Methods

Reagents and chemicals

Tetramine and desipramine-D3 were purchased from Cerilliant Corporation (Round Rock, Texas, USA). All the solvents were of High-Performance Liquid Chromatography (HPLC) grade. Ammonium formate and formic acid were obtained from Fluka (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (MT, USA). Deionized water was produced using an Elga Purelab Option-Q ultra-pure water system (Lane End, UK).

Sample preparation

A simple protein precipitation method was used to extract tetramine. Blood and urine samples (0.1 ml) were taken and spiked with 30 µl of a working solution of the internal standard (1 mg/L of desipramine-D3). To each, 0.3 ml of acetonitrile was added, and the solutions were vortexed for 30 s. Thereafter, they were subjected to ultrasonic waves for 15 min. After centrifugation at 12,000 rpm for 10 min, the supernatants were filtered with a 0.22 µm polyvinylidene fluoride membrane. Aliquots (5 µl each) of the filtrates were injected into the LC-MS/MS system with the MRM mode show in Figure 1.

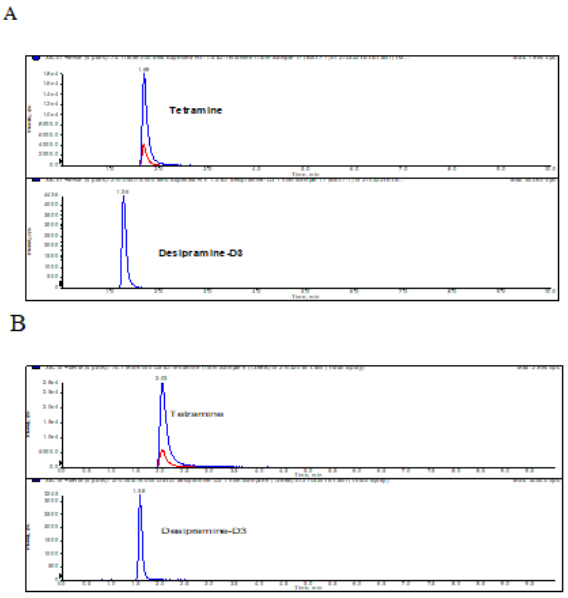

Figure 1: Representative chromatograms of, A) Tetramine and desipramine-D3 in fortified (B) Femoral blood sample.

LC-MS/MS analysis

The LC-MS/MS analysis was performed on Agilent 1290 Infinity UHPLC system consisting of a pump, auto sampler, on-line degasser, and column compartment (Agilent Technologies, CA, USA), (AB SCIEX QTRAP 4500 MS/MS) (AB SCIEX, MA, USA). Chromatographic separation was performed in 12 min. The analytical column used was Syncronis Hilic (2.1 × 100 mm, 1.7 µm, Thermo Fisher Scientific, MA, USA) which was maintained at 40°C. The temperature of the auto sampler was maintained at 10°C. The MS system was operated using Electro Spray Ionization (ESI) in positive mode. The individual MRM transitions, retention times, and other experimental parameters are shown in Table 1.

Compound	Precursor ion	Product ion	RT (min)	DP	EP	CE	CXP
	(m/z)	(m/z)					
Tetramine	74.12	59/58.2	1.5	46	10	10	21
				46	10	10	29
Desipramine-D3	270.4	116	1.2	116	10	10	21

Table 1: MRM transitions, retention times and conditions for tetramine and desipramine-D3. RT: Retention Time; DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential.

The mobile phases consisted of 2.5 mm ammonium formate containing 0.1% formic acid in water (mobile phase A) and 2.5 mm ammonium formate containing 0.1% formic acid in acetonitrile (mobile phase B). For gradient elution, the mobile phase B was maintained at 95% for 3 min. It was subsequently decreased to 10% till 7 min and then maintained for 1 min. Finally, the initial condition was restored and held for 4 min to re-equilibrate the system. The total run time was 12 min. The final injection volume was 2 µl. For the mass spectrometer, AB SCIEX's 4500 QTRAP, equipped with a turbo ion spray was used. For the ionization conditions, an analysis in the ESI positive mode was conducted. The Multiple Reaction Monitoring (MRM) mode analytical conditions for the detection and quantitation of the target drugs were determined using ANALYST software in the optimization mode.

Validation of methods

The following parameters were evaluated: linearity, matrix effect, recovery, process efficiency, the Limit of Detection (LOD), Limit of Quantitation (LOQ), precision, and accuracy. The analytical method was validated using spiked blood and urine samples. We analyzed the blood and urine samples in replicates of five using six samples of tetramine at concentrations ranging from LOQ (25 mg/ml-200 mg/ml), and then we used the results to prepare the calibration curves. The LOD and LOQ were determined by analyzing blank blood and urine samples fortified with known drug concentrations. Each concentration was measured in five replicates. The LOD was defined as the lowest concentration giving a response of three times the average baseline noise defined from five unfortified samples. The LOQ was defined as the lowest observed concentration for 10 replicates with less than ± 20% Coefficient of Variation (CV) for precision and less than ± 20% for bias. The recovery, process efficiency, and matrix effect were evaluated at two Quality Control (QC) concentration levels (low and high) as proposed by Matuszewski et al. [7]. The evaluation was done by comparing analytic peak areas of neat standards and five blood and urine samples extracts fortified with QC solutions before and after extraction at low and high concentrations. The accuracy and precision (presented as the CV) were evaluated by analyzing the QC samples at three concentrations. The accuracy (%) was determined from the percentage ratio of the measured nominal concentration (mean of measured/nominal × 100). The intra-day CV and accuracy of the method were evaluated by analyzing five replicates (n=5) of the samples. The CV and accuracy of the inter-day assays were evaluated by analyzing five replicates of the samples on five different days (n=25).

Results and Discussion

General toxicological analysis was performed on the blood, and urine samples to screen for other chemical substances such as cyanide, agrochemicals, narcotics, medicines and natural toxins. No other chemicals except tetramine were detected in the two patient's urine and blood samples. A protein precipitation and quantitative LC-MS/MS method for the detection of tetramine in blood and urine samples was fully developed and validated. The chromatographic separation was completed within 12 min. The LOD and LOQ of tetramine in the blood and urine samples were 5 mg/ml and 25 mg/ml, respectively. The correlation coefficients (r2) of the blood and urine samples were 0.995 and 0.998, respectively. The CVs (%) and bias (%) were below 15% in intra and inter-assays (Table 2).

	Concentration	Intra-assay		Inter-assay	
	(ng/mL)	Precision	Accuracy	Precision	Accuracy

		(CV, %)	(bias, %)	(CV, %)	(bias, %)
Blood	50	0.6	4.1	1.15	5.12
	100	1.6	–5.32	1.28	–2.86
	200	0.97	–10.22	1.11	3.53
Urine sample	50	1.69	4.45	7.17	–5.70
	100	8.25	2.2	1.64	–1.40
	200	1.43	2.45	2	–1.74

Table 2: Precision and accuracy of tetramine in blood and urine samples.

The results of the matrix effect, recovery, and process efficiency of tetramine and desipramine-D3 in the blood and urine samples are summarized in Table 3.

		Concentration	Matrix effect		Recovery		Process efficiency	
		(ng/mL)	Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)
Blood	Tetramine	100	55.8	3.1	99.1	1.2	55.4	3.2
		200	71.0.	1	92.6	5.2	65.8	6.2
	Desipramine-D3	30	107.3	3	99.9	2.7	107.2	2.8
Urine sample	Tetramine	100	75.5	2.9	106.2	1.3	80.2	3.8
		200	92.4	1.5	102.3	3.4	94.5	3.7
	Desipramine-D3	30	98.6	2.5	106.4	2.6	104.9	3.3

Table 3: Matrix effect, recovery and process efficiency of tetramine and desipramine-D3.

The mean values and CVs (%) of the matrix effect, recovery, and process efficiency showed satisfactory results.

Amounting evidence suggests Neptunea as a major cause of tetramine poisoning. In a previous report, tetramine contents in the salivary gland of edible whelk *Neptunea antiqua* was 5.7 mg/g (range: 4~9 mg/g of the gland) [1]. An Irish study reported that a clearly defined seasonal cycle of tetramine concentration in the whelk's salivary gland; the concentration ranged from undetectable levels to 6.5 mg/g during an annual sampling exercise in the Irish Sea, and the content of tetramine increased progressively over the summer and autumn to reach a peak in the winter months [8]. A study on the content of tetramine in Neptunea living on the Korean coast reported the presence of tetramine *in N. arthritica, N. arthritica cumingfii, Buccinum striatissinum, and Volutharpa perryi* [9]. The effects of tetramine toxin on the digestive system include vomiting, nausea, and stomach ache. In addition, tetramine toxin causes headache, dizziness, blurred vision, paralysis, and ataxia [4,10]. The minimal content of tetramine that causes toxic effect was estimated to be above 10 mg, which is the amount ingested by eating 2-4 shellfish [1,3].

In the present study, the concentration of tetramine in the husband's blood was higher than that of the wife; the concentrations of tetramine in the husband's femoral vein blood and urine were 1.37 mg/L and 15.07 mg/L, respectively, and 0.57 mg/L and 5.85 mg/L, in the wife's blood and urine respectively (Table 4). We found that 0.57 mg/L and 1.37 mg/L of tetramine in the blood exerted sufficient toxicity with symptoms including mental stupor and semi-coma state. Since few studies have reported toxic and fatal levels of tetramine in the blood, this study can be a reference to evaluate tetramine poisoning in the clinical and postmortem toxicology.

	Blood (mg/L)	Urine (mg/L)
Male	1.37	15.07
Female	0.57	5.85

Table 4: Concentrations of tetramine in blood and urine samples from two intoxication cases.

Conclusion

In this study, we developed and validated a method that combined protein precipitation and LC-MS/MS. We performed a quantitative analysis of tetramine in blood and urine samples from two patients transferred to a hospital emergency room. Our findings confirmed that this method was sensitive and selective in the detection and quantification of tetramine in blood and urine samples. Tetramine neurotoxicity is fatal, and therefore, prompt determination of tetramine in patients' samples is important in guiding proper and timely treatment. The developed analytical method would be beneficial in the clinical and forensic toxicology fields.

Conflict of Interest

There are no financial or other relations that could lead to a conflict of interest.

Acknowledgment

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