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PAPER

TOXICOLOGY

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Hair as a Specimen to Document Tetramethylene Disulfotetramine Exposure*

ABSTRACT: Tetramethylene disulfotetramine (tetramine) is a rodenticide that has been banned for many years in China. Since 2005, inhabitants of a village in the Henan Province have been suffering from grand mal seizures. To investigate the possibility of tetramine as the cause, we developed a method to determine tetramine in human hair. Sample preparation involved external decontamination, frozen pulverization, and ultrasonication in 2 mL ethyl acetate in the presence of cocaine-d3 as an internal standard. The method exhibited good linearity; calibration curve was linear over a range of 0.1–20 ng/mg hair. The limit of detection for the assay was 0.05 ng/mg hair. Except for one subject (No. 4), all head and pubic hair samples were positive for tetramine. The concentrations of tetramine in pubic hair were significantly higher than those in the same subjects' head hair samples. Because of a long retention in body, segmental head hair analysis cannot provide an accurate exposure history of tetramine in the body.

KEYWORDS: exposure, forensic science, forensic toxicology, GC-MS, hair, tetramethylene disulfotetramine

Tetramethylene disulfotetramine, also known as tetramine, is a highly toxic rodenticide. Tetramine acts directly on the brain itself, blocking the chloride channel of the inhibitory neurotransmitter receptor of γ-amino butyric acid (GABA) (1). Very small amounts of tetramine, estimated as low as 0.1 mg/kg weight (oral LD_{50}) in mammals, can lead to convulsions and death, with 7.0–10.0 mg/kg dosages considered lethal in humans (2); the minimum lethal dose for humans has been estimated at 5.0 mg/kg. The effects of tetramine can manifest themselves quickly, from approximately 30 min to a few hours after ingestion (3,4). Mild symptoms include headache, dizziness, fatigue, anorexia, nausea, vomiting, numbness of lips, and listlessness. Severe symptoms include loss of consciousness, seizures (grand mal epilepsy type, or lasting 1–6 min and occurring repetitively after several minutes), foaming at the mouth, urinary incontinence, coma (with ECG/EEG abnormalities), and death from respiratory failure (3,4). Retention of tetramine in the tissues of poisoned poultry and animals also poses the risk of secondary acute intoxication in humans postingestion. Tetramine has been banned for many years in China (1,5), but is still in use in certain areas, especially in the countryside.

Blood, urine, kidneys, liver, and other biological tissues are the conventional specimens to document tetramine poisonings (2,4,6). Tetramine can be detected by gas chromatography-mass spectrometry (GC-MS) and GC with nitrogen/phosphorous detection (2,4,6–11). Unfortunately, no data are available for testing tetramine in hair, which could be considered a specimen of choice to complement blood and urine analysis in cases of chronic intoxication.

We present here a developed method to quantify tetramine in hair by GC-MS and its application to a case involving more than eight subjects.

Case History

Since 2005, many people living in a village in Henan Province have been suffering from grand mal seizures. They were taken to the hospital and eventually recovered. Several months after they returned home, some patients again developed severe symptoms including loss of consciousness and grand mal seizures. One patient even died. The villagers were convinced that their village was haunted.

When these rumors reached the government, the police began to investigate. On July 17, 2009, crop, soil, and water sources for the village were collected and sent to a local laboratory in the Henan Province Nanyang Public Security Bureau. All samples came back as negative under the current screening method. On July 29, 2009, more samples including blood, flour, vegetables, and dust from the victims' homes were collected and sent to the same laboratory. Several floor dust samples collected from the victims' living rooms, kitchen, and bedrooms tested positive for tetramine. No tetramine was detected in blood from the victims, however. The laboratory was requested to analyze hair strands collected on September 25, 2009, to evaluate the possibility of tetramine as the cause of the intoxication. Two control hair specimens were collected on October 8, 2009. The collected hair strands were then used in the following analysis.

Materials and Methods

Chemicals

Tetramine (chromatographic purity, 99%) and cocaine-d3 (chromatographic purity, 99%) were purchased from Cerilliant (Round

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Rock, TX). High-performance liquid chromatography grade methanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate and acetone were of analytical grade. Deionized water was purified using a Milli-Q system (Millipore, Billerica, MA).

Standard Solutions

Stock solutions were purchased at following concentrations: 100 μg/mL tetramine and 1 mg/mL cocaine-d3. A tetramine working solution was prepared at 10 μg/mL in acetonitrile and further diluted with acetonitrile to yield working solutions for calibration and QC samples. An internal standard (IS) working solution of cocaine-d3 was prepared at 10 μg/mL in acetonitrile. Working solutions were prepared monthly and stored at 4°C.

Specimens

Hair samples were collected from villagers in the village. Hair was cut with round-point scissors from the posterior vertex as close to the scalp as possible, the scalp-end was labeled, and the hair was stored in clean paper bag at room temperature. Blank hair samples were obtained from healthy people in the laboratory.

Sample Preparation

When the length of head hair was more than 4 cm, hair was segmented (3-cm size/segment) axially from the scalp-end prior to decontamination and was treated as a separate sub sample. Approximately 100 mg of head hair segments and pubic specimens were washed with different solvents as follows: 5 mL of 0.1% sodium dodecyl sulfate for 5 min, twice with 5 mL of deionized water for 5 min, and twice with 5 mL of acetone for 5 min. The last acetone washing solution was collected, evaporated, and reconstituted in 50 μL of ethyl acetate, and analyzed by conventional GC-MS procedures to exclude contamination (http://www.soht.org/html/Statements.html) (12). After being air-dried, the segments were cut into small pieces of <3 mm and pulverized in a 6770 SPEX CertiPrep freezer mill (Metuchen, NJ). A total of 50 mg of powered hair was sonicated in an ultrasonic bath in 2 mL of ethyl acetate for 1 h at room temperature, with 200 ng cocaine-d3 added as an IS. After ultrasonication, the samples were centrifuged, and the supernatant was evaporated under nitrogen gas on a heated metal block at 50°C. The residue was reconstituted in 50 μL of ethyl acetate, and a 1 uL aliquot of the solution was injected into the GC-MS system running in selected ion-monitoring (SIM) mode.

Instrumentation

GC-MS analyses were carried out on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973 mass spectrometer, and an Agilent 6890 series automatic injector (Agilent Technologies, Palo Alto, CA).

The analytical column was an HP-5MS capillary column (5% phenyl methyl siloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA).

The temperature programs were applied as follows: the oven temperature was held at 100°C for 1.5 min, then increased to 280°C at 25°C/min and held for 5 min, giving a total run time of 13.7 min. The temperatures for the injection port, ion source, and transfer line were set at 280, 230, and 280°C, respectively. Injection was carried out in splitless injection mode, and the flow of the carrier gas (helium) was maintained at 1.0 mL/min in constant flow mode.

The mass detector was operated at 70 eV in electron impact SIM mode. The following diagnostic ions were monitored for each compound: m/z 212 (base peak) and 240 (55% base peak intensity) for tetramine; and m/z 185 and 306 for cocaine-d3. For tetramine identification, ion ratios should be within ± 20% relative to that of the standard analyte (http://www.soft-tox.org/files/Guidelines_2006_Final.pdf). The ions m/z 212 and 185 were used for quantitative determinations.

Analytical Method Validation

The method was validated according to the procedures of Peters et al. (13). A one-way analysis of variance was performed with Stata 7 software (STATA, College Station, TX).

To investigate the sensitivity and potential background interference of the method, 10 sources of blank hair powder from healthy people in our laboratory were analyzed.

Blank powered hair (n = 2 at each concentration level) were fortified to obtain calibration standards with concentrations of 0.1, 0.2, 0.5, 2, 10, and 20 ng tetramine/mg hair. Each calibration standard was also fortified with 20 μL of the IS solution containing 10 μg/mL cocaine-d3. The calibration standards were extracted as unknown samples. A six-point standard curve was prepared by a linear least square regression analysis of the peak area ratios of tetramine to the IS versus analyte concentrations. Daily calibration curves using the same concentrations (n = 1 at each concentration level) were prepared for all experiments.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing decreasing concentrations of tetramine added to drug-free hair and extracted as described. The LOD was defined as the lowest concentration that produced a response that was three times higher than the background noise. The LOQ was defined as the lowest point on the calibration curve that fulfilled the LOQ criteria based on precision and accuracy data: <20% for precision and ±20% for accuracy.

Recoveries were established at low, medium, and high concentrations by comparing the analyte peak areas of fortified extracted samples (n = 6) to those of pure standards fortified with the same amounts of tetramine.

Accuracy and precision were determined using blank hair powder fortified with tetramine at low, medium, and high concentrations (0.2, 2, and 20 ng/mg, respectively) relative to the calibration range. The intra-day precision was determined by assaying six fortified hair samples at each concentration level on the same day, and six replicates on each of 4 days were assayed for inter-day precision. The concentrations were calculated via the daily calibration curves. Accuracy was established by assaying six replicates at three concentrations and expressed as the percentage of the determined concentration compared to their fortified concentrations.

Results

Method Validation

Assay selectivity was confirmed by the absence of interfering peaks at the retention times for tetramine in blank hair powders. Figure 1 shows the chromatograms of samples of blank hair, hair spiked at the level, of the lowest calibration level, and a positive sample.

The calibration curve was constructed by plotting the area ratio of tetramine and the IS against fortified concentrations. The resulting calibration curve for tetramine was $y = 0.0454 \times -0.0014$, $R^2 = 0.9995$, and exhibited good linearity. The LOD and LOQ were 0.05 ng/mg and 0.1 ng/mg, respectively.

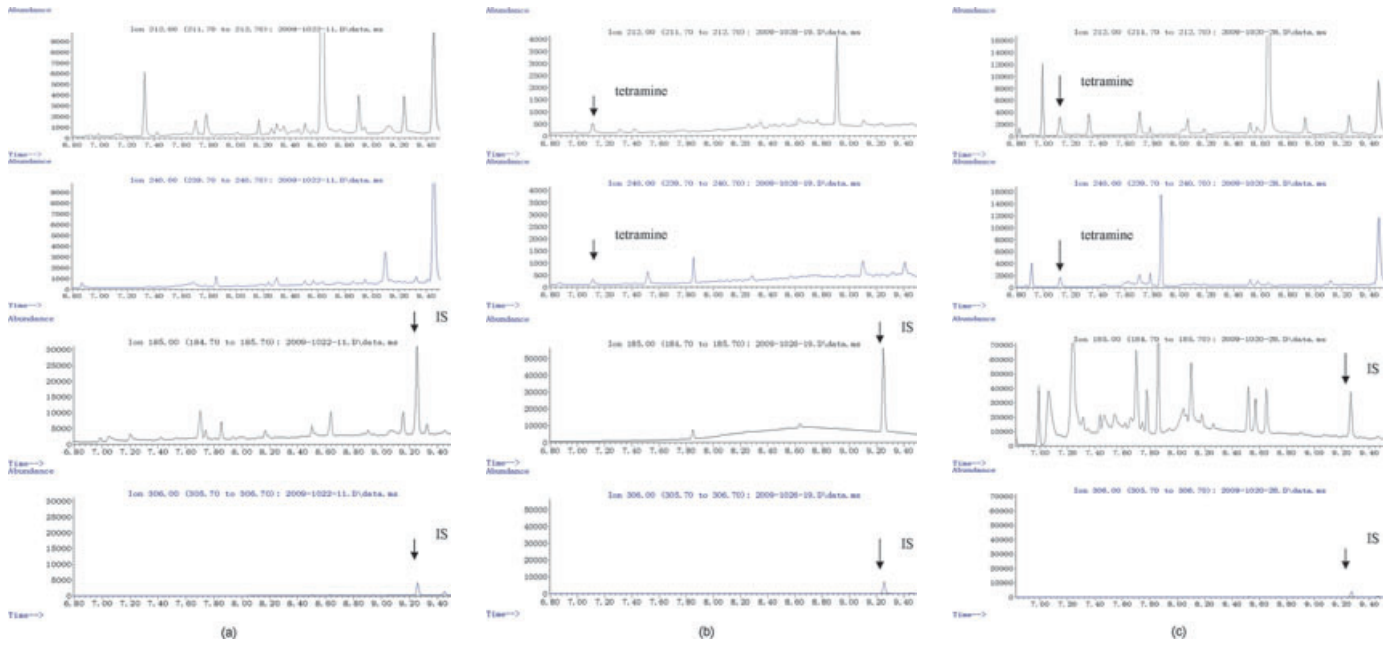

FIG. 1—*(a) Chromatograms of a blank sample with internal standard; (b) chromatograms of tetramine at the LOQ concentration; and (c) the positive result of tetramine in a victim's pubic sample (measured concentration: 0.74 ng/mg).*

Extraction recoveries were determined by comparing the peak areas of extracted standards to those of neat standards ($n = 6$) at low and high concentrations. For tetramine, these were 66.71% and 84.59% at 0.2 ng/mg and 20 ng/mg, respectively.

The analytical precision and accuracy were evaluated at three concentrations (low, middle, and high) covering the linear dynamic range of tetramine. The results of the intra- and inter-day precision and accuracy experiments at all three concentrations are given in Table 1 and ranged from 0.80 to 3.85% and from 1.19 to 6.47%, respectively. Overall, the accuracy of the method at the three concentrations and the extraction recoveries were acceptable.

Case Study Results

The analytical results of eight victims and two control subjects from four families are presented in Table 2 along with case details. With an LOD of 0.05 ng/mg, no tetramine was detected in the last acetone wash of the method. Except for one subject, No. 4, and two control samples (Subject No. 9 and Subject No. 10), all head and pubic hair samples tested positive for tetramine. The concentrations of tetramine in the segments near the ends of hair were higher than in the proximal segments, and the concentrations of tetramine in pubic hair were significantly higher than in the same subject's head hair samples.

TABLE 1—*Accuracy and precision of the method.*

Analyte	Fortified Concentration (ng/mg)	Accuracy (% bias) ($n = 6$)	Precision (%) Intra-Day ($n = 6$)	Precision (%) Inter-Day ($n = 24$)
Tetramine	0.2	14.38	3.85	6.47
	2	−1.76	0.80	3.42
	20	0.03	1.91	1.19

Discussion

Tetramine has been banned in China since 1984 because of its human toxicity, but some previously purchased quantities are still scattered among consumers, particularly in rural areas. As an effective and popular rodenticide, tetramine may have contaminated the victims' living rooms, kitchens, or bedrooms several years ago, even though the villagers did not intentionally mean to do any harm. The most common route of exposure is ingestion of contaminated foods. Despite its unique symptom of convulsions, tetramine poisoning can often be difficult to diagnose. To our knowledge, the main reasons for this difficulty are that victims typically cannot provide any useful exposure history, symptoms are often confused with epilepticus, and laboratory identification of the compound is not practical for acutely poisoned patients in local Chinese hospitals.

The ideal IS possesses similar physicochemical properties to the analyte, making isotopically labeled standards ideal. Tetramine, however, does not currently have any deuterated analogs on the market, and it is difficult to find a suitable IS standard because of its unique chemical structure (Fig. 2). The use of pesticides as tetramine ISs has been described in the literature (7,14), but cocaine-d3 was selected for the current study based on its lipophilicity, GC retention time, and isotopic labeling.

Surface exposure contamination was a significant problem for hair analysis in this case study. Until now, there has been no data on the detection of tetramine metabolites. To minimize the possibility of external contamination causing any misinterpretation, we first decontaminated the hair samples. No tetramine was detected in the last acetone wash of the method, given the LOD of 0.05 ng/mg. Two control hair samples (Subject No. 9 and Subject No. 10) were sent to our laboratory and tested negative for tetramine. Subject No. 9 and Subject No. 10 were both 7 years old and lived in the same village. Subject No. 9 was from family #4, while Subject No. 10 was from family #1. They were both in good health and played together with Subject No. 7 on June 28, 2008. Additionally, a cut-off level can be used to exclude external contamination (12),

TABLE 2—*Hair sample results and case details.*

Family	Subject(No.)	Sex	Age	Hair Sample	Results (ng/mg)	Case Details
#1	1	M	42	Head: 4 cm	0.25	1. Suffered a grand mal seizure in November 2006.
				Pubic: 6 cm	1.55	2. Physical symptoms occurred again in July 2008.
						3. Symptoms occurred again in March 2009. At that time, tetramine was detected in blood by the Henan Province CDC Laboratory. Blood specimens collected on July 29, 2009 and September 25, 2009 tested negative for tetramine.
	2	F	43	Head: 0–3 cm	0.22	1. Suffered a grand mal seizure in July 2008. Blood specimens collected on July 29, 2009 and September 25, 2009 tested negative for tetramine.
				3–8 cm	0.64	
				Pubic: 5 cm	0.74	
#2	3	M	51	Pubic: 5 cm	1.2	1. Suffered a grand mal seizure in January 2008.
						2. Physical symptoms occurred again in July 2009.
	4	F	49	Pubic : 4 cm	0.24	1. Symptoms occurred in January 2008. Blood specimen collected on July 29, 2009 tested negative for tetramine.
				Head : 0–3 cm	–	
				3–6 cm	0.26	
#3	5	M	37	Pubic: 4 cm	0.98	1. Symptoms occurred in September 2008. Blood specimen collected on July 29, 2009 tested negative for tetramine.
	6	F	36	Pubic: 4 cm	0.21	1. Physical symptoms occurred in August 2008. Blood specimen collected on July 29, 2009 tested negative for tetramine.
	7	M	7	Head: 0–3 cm	0.33	1. Suffered a grand mal seizure in June 2008. Blood specimen collected on July 29, 2009 tested negative for tetramine.
				3–6 cm	0.35	
				6–11 cm	0.51	
	8	F	5	Head: 4 cm	0.89	1. Physical symptoms occurred in August 2008. Blood specimen collected on July 29, 2009 tested negative for tetramine.
#4	9	M	7	Head: 2 cm	–	1. Control group. Living in the same village with different surnames. Played with Subject No. 7 on June 28, 2008. In good health. Hair specimen was collected on October 8, 2009.
#1	10	F	7	Head: 0–3 cm	–	1. Control group. Played with Subject No. 7 on June 28, 2008. In good health. Hair specimen was collected on October 8, 2009.
				3–6 cm	–	
				6–9 cm	–	
				9–27 cm	–	

–: not detected.

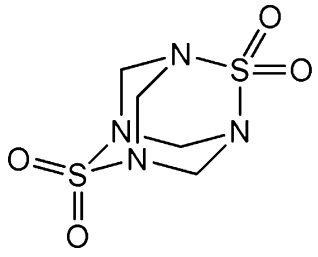

FIG. 2—*The chemical structure of tetramine.*

but our small sample size and tetramine's long retention time in body make this difficult to execute.

The results in Table 2 demonstrate that tetramine can enter into hair. The degree of the drug's disposition in hair closely relates to its affinity with melanin and lipophilicity (15). All head and pubic hair segments were black, so there was no significant difference in pigmentation. Tetramine has a cyclical or ring-like molecular structure (16) and is lipophilic enough to penetrate membranes and diffuse from blood capillaries into growing cells between the matrix cells and end of the keratinization zone of the hair follicle (17). Tetramine can also be deposited into hair by diffusion from sweat or sebum secretions into the completed hair shaft. Tetramine is stable in a wide variety of matrices (7,18) and is not easily degraded. Once incorporated into hair, concentrations of tetramine declined slowly along the hair shaft.

Pubic hair concentrations of tetramine were significantly higher than in the same subjects' head hair samples. Similar results have been reported for opiates (19), amphetamines (20), and benzodiazepine (21). This high concentration may be caused by the continuous urinary contamination of pubic hair and a different growth rate of hair in this area (19,20). Additionally, there were substantial inter-site differences in hair growth rates and cycles, in the nature and activity of sweat and sebum glands, and in hair thickness (22).

The practical advantage of hair testing over urine or blood testing for drugs is that it has a larger surveillance window (23,24). Multisectional analysis can provide a retrospective calendar of an individual's drug use. The proximal 0–8 cm segments should be negative for drugs if the person had only been exposed once within the last year, considering hair growth of 0.7–1.4 cm per month (25). However, head hair results for the case were not consistent with this regular pattern. Retention of tetramine in the body was responsible for the large, broad band of tetramine in the hair shaft. It has also been demonstrated that tetramine distributes quickly in all body tissues and fluids and eliminates from the body slowly (4). We have found that urine can test positive for tetramine for up to 6 months after exposure (4,6). Barrueto et al. (26) reported on a 15-month-old female who was exposed to tetramine by accidental ingestion. Six months after exposure, the child remained severely developmentally delayed and required pharmacological therapy for seizure control. Even if tetramine has been eliminated through body fluids, it may still remain in the sebum glands and is released slowly because of its lipophilicity.

Conclusions

A sensitive and specific method for the determination of tetramine in human hair was developed and validated. Hair samples should prove useful in documenting tetramine exposure. The concentrations of tetramine in pubic hair were significantly higher than

in the same subject's head hair samples. Because of its long retention in body, segmental head hair analysis cannot provide an accurate exposure history for this drug.

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