TECHNICAL NOTE

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Determination of Phenmetrazine in Urine by Gas Chromatography-Mass Spectrometry after Liquid-Liquid Extraction and Derivatization with Perfluorooctanoyl Chloride

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ABSTRACT: Phenmetrazine is a central nervous system stimulant and is currently used as an anorectic agent. The drug is abused and reported to cause death from overdose. We describe a liquid-liquid extraction protocol for phenmetrazine from urine using 1-chlorobutane and subsequent derivatization using perfluorooctanoyl chloride for gas chromatography-mass spectrometric confirmation. Quantitation of urinary phenmetrazine can be easily achieved by using N-propylamphetamine as an internal standard. The perfluorooctanoyl derivative of phenmetrazine showed a weak molecular ion at m/z 573 and a characteristic strong peak at m/z 467 in the electron ionization mass spectrometry thus aiding unambiguous identification. The perfluorooctanoyl derivative of the internal standard did not show any molecular ion, but showed strong characteristic peaks at m/z 482 and 440. The within run and between run precisions of the assay were 1.7% and 3.2% at a urinary phenmetrazine concentration of 20 μg/mL. The within run and between run precisions were higher (9.4% and 10.8%) at a urinary phenmetrazine concentration of 1.0 μg/mL, which was very close to the detection limit of the assay. The assay was linear for urinary phenmetrazine concentration of 1 to 100 μg/mL with a detection limit of 0.5 μg/mL.

KEYWORDS: forensic science, forensic toxicology phenmetrazine, gas chromatography-mass spectrometry, perfluorooctanoyl

Phenmetrazine (preludin) is the N-desmethyl analogue of phendimetrazine. The drug is used as an anorectic agent and is available for oral use as a hydrochloride salt in single dose of 25 mg and daily doses up to 75 mg (1). The drug has a high potential for abuse and in some countries like Sweden, the drug was withdrawn from the market. Phenmetrazine overdose can cause dizziness, tremor, tachycardia, hypertension, cardiac arrhythmia, convulsion, coma, and circulatory collapse. Several deaths have been reported from phenmetrazine overdose. Norheim reported a urinary phenmetrazine concentration of 24 μg/mL in a fatal case caused by intravenous use of the drug (2). Gottschalk reported an average urinary phenmetrazine concentration of 21 μg/mL in 12 fatalities. The highest reported concentration in that paper was 90 μg/mL of urinary phenmetrazine. Cravey reported a urinary phenmetrazine concentration of 2.5 μg/mL in a person who committed suicide by gunfire (1). In another report the urinary phenmetrazine concentration varied between 56 to 290 μg/mL in seven drug users (3).

Phenmetrazine cross reacts with the EMIT assay for urinary amphetamines only at higher concentrations and, therefore, an initial screen of urine using EMIT dau kit may be negative even for significant concentration of urinary phenmetrazine. There are only a few reports in the literature dealing with gas chromatography-mass spectrometric (GC/MS) identification and quantitation of urinary phenmetrazine. Phenmetrazine can be analyzed without derivatization. Beckett et al. described a protocol for flame ionization gas chromatographic determination of underivatized drug (4). However, the molecular weight of underivatized phenmetrazine is only 177 and the peak suffered from tailing. Therefore, other investigators derivatized phenmetrazine prior to analysis. Franklin et al. described an N-acetyl derivatization of the drug (5). Recently, Kronstrand et al. described a GC/MS protocol for the determination of phenmetrazine in urine after derivatization with methyl chloroformate (6). The authors took advantage of their previously described protocol for derivatization of amphetamines using methyl chloroformate (7) and used ethylamphetamine as in internal standard.

Phenmetrazine has a morpholine ring to a lactum and the morpholine ring appears to be metabolically stable. Therefore, a derivatization reaction under condition that is somewhat more stringent than carbamate formation may not hydrolyze the lactone ring. We studied the possibility of forming a perfluorooctanoyl derivative of phenmetrazine for GC/MS analysis. The molecular weight of phenmetrazine methyl carbamate is 235, while the molecular weight of the perfluorooctanoyl derivative of phenmetrazine is 573. Therefore, the perfluorooctanoyl derivative of phenmetrazine may produce more characteristic ions at a higher mass range for unambiguous confirmation and lesser volatility of the derivative may aid in avoiding interferences from more volatile components of the urinary matrix. Recently Gjerde described derivatization of amphetamines using perfluorooctanoyl chloride (8). Here we describe GC/MS confirmation and quantitation of urinary phenmetrazine after derivatization with perfluorooctanoyl chloride.

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Materials and Method

Phenmetrazine, the internal standard N-propylamphetamine, amphetamine, methamphetamine, pseudoephedrine, phentermine, phenylpropanolamine, MDMA and MDPA were obtained from Altech Applied Science (College Park, PA). The derivatizing agent perfluorooctanoyl chloride was obtained from PCR (Gainesville, FL). HPLC grade 1-chlorobutane (extraction solvent), sodium tetraborate decahydrate, and sodium hydroxide were purchased from Aldrich (Milwaukee, WI). We prepared the internal standard (propyl amphetamine) in methanol (1 mg/mL). The EMIT assay kit for urinary methamphetamine was obtained from Behring diagnostics (San Jose, CA) and the assays for determining cross reactivity of urinary phenmetrazine with amphetamine were run on the SYVA-30 R automated analyzer also obtained from Behring diagnostics.

To extract phenmetrazine from urine, we supplemented a 2-mL aliquot of urine with 20-μL of the internal standard solution (the final concentration of the internal standard in urine was 10 μg/mL), followed by the addition of 1-mL borate buffer (pH 9.8) and 1-mL 1N sodium hydroxide. The borate buffer was prepared by dissolving 20 g of sodium tetraborate decahydrate in 1L of deionized water. Phenmetrazine along with the internal standard were extracted from the alkaline urine using 10 mL of 1-chlorobutane. We vortex mixed the sample for 1 min and then further mixed the sample in a rotating mixture for an additional 10 min. After centrifugation for 5 min at 1500 g, the upper organic layer was transferred to a conical test tube and the organic phase was almost evaporated to dryness (approximately 50 μL of organic phase remained). Then 50 μL of the derivatizing agent (perfluorooctanoyl chloride) was added to the remaining organic phase. After heating the reaction mixture at 80°C for 30 min, the reaction mixture was evaporated to dryness and the residue was reconstituted with 50 μL of ethyl acetate. Then 2 μL of the reconstituted mixture was injected into the GC/MS for further analysis.

The gas chromatography-mass spectrometric analysis was carried out by using a model 5890 series II gas chromatograph coupled to a 5972 mass selective detector (Hewlett Packard, Palo Alto, CA). We used an Ultra 1 capillary column (25 m by 0.2 mm), which was coated with cross linked methyl silicone (0.33 μm film thickness). The initial oven temperature of the gas chromatograph was 120°C. After maintaining that temperature for 5 min, the oven temperature was increased at a rate of 20°C/min to reach an oven temperature of 280°C. The final temperature was maintained for an additional 1 min. The run time was 14 min with a solvent delay time of 7 min. The injector port temperature was 250°C and we used splitless injection. The mass spectrometer was operated in the electron ionization mode (scanning range: m/z 50–700).

The quantitation was done by comparing the area under the peak for phenmetrazine with the area under the peak of the internal standard, N-propylamphetamine. The spectra were obtained using the scan mode.

Results and Discussion

Cross Reactivity of Phenmetrazine with the EMIT Assay for Urinary Amphetamines

Since many laboratories screen urine specimens for suspected drugs of abuse by the EMIT immunoassays, we studied the possible cross reactivity of phenmetrazine with the EMIT assay for amphetamines. We prepared several standard urine specimens containing phenmetrazine by supplementing drug free urine (negative by the EMIT screen) with the phenmetrazine standard solution prepared in methanol (1 mg/mL). We observed no cross reactivity (EMIT screen negative for amphetamines) with urinary phenmetrazine concentrations of 1 and 5 μg/mL, respectively. However, the 10 μg/mL standard was strongly positive for amphetamines. We further studied the range of concentrations of phenmetrazine from 5 to 10 μg/mL and determined that a urine containing 6.6 μg/mL should be screened as positive by the EMIT assay for amphetamine. In another experiment, a urine specimen supplemented with 6.2 μg/mL of phenmetrazine was screened as positive by the EMIT assay for amphetamines. A urine specimen containing 1 μg/mL of amphetamine would be screened as positive by the EMIT assay. The manufacturer reported that a specimen containing 6.0 μg/mL of phenmetrazine should be screened as positive by the assay. Therefore, our observation is in agreement with that of the manufacturer. A slight lot-to-lot variation in determining the positive screen by the EMIT assay in specimens containing phenmetrazine is also possible. Because phenmetrazine showed positive only at a high concentration, urine specimens containing low to moderate amounts of phenmetrazine may be negative by the EMIT assay. Therefore, an effective GC/MS assay for confirmation and quantitation of phenmetrazine in urine which is sensitive to low urinary concentrations of phenmetrazine and at the same time can effectively analyze high concentrations would be useful.

Chromatographic Properties of Derivatized Phenmetrazine

We used 1-chlorobutane for our liquid-liquid extraction because Hornbeck et al. used the same extraction solvent in the final stage of the extraction process for amphetamines (9). Use of 1-chlorobutane for extraction of amphetamines had also been documented in earlier reports (10,11). Kronstrand et al. used isooctane for extraction of phenmetrazine from urine (6). Isooctane has a higher boiling point than 1-chlorobutane and requires a longer time for concentrating the organic extract. Moreover, we evaporated the solvent at room temperature in order to avoid loss of phenmetrazine and the internal standard; 1-chlorobutane is a better solvent due to its higher volatility.

We observed base line separation between the derivatized internal standard and phenmetrazine. The perfluorooctanoyl derivative of N-propy amphetamine (internal standard) eluted at 9.7 min while the perfluorooctanoyl derivative of phenmetrazine eluted at 10.3 min. The typical total ion chromatogram of a urine specimen supplemented with 20 μg/mL of phenmetrazine is given in Fig. 1.

Mass Spectral Characteristics of Perfluorooctanoyl Derivative of Phenmetrazine

Phenmetrazine is a small molecule with a molecular weight of 177. In our new derivatization protocol, the molecular weight of the perfluorooctanoyl derivative of phenmetrazine is 573. We observed a weak molecular ion at m/z 573 (relative abundance; 0.8%) in the mass spectrum. A strong characteristic peak at m/z 467 (relative abundance: 34.8%) was observed due to fragmentation in the lactone ring of the molecule which retained the perfluorooctanoyl group. Another characteristic peak was observed at m/z 176 (relative abundance: 8.3%) due to loss of the perfluorooctanoyl group from the derivatized phenmetrazine. The base peak was observed at m/z 70 (Fig. 2). The base peak was similar to that observed in methyl phenmetrazine carbamate.

We observed an extremely weak molecular ion in the perfluorooctanoyl derivative of N-propyl amphetamine (relative abundance: 0.5%), but very strong characteristic peaks were present at m/z 482 (relative abundance: 100%) and 440 (relative abundance: 99%). We

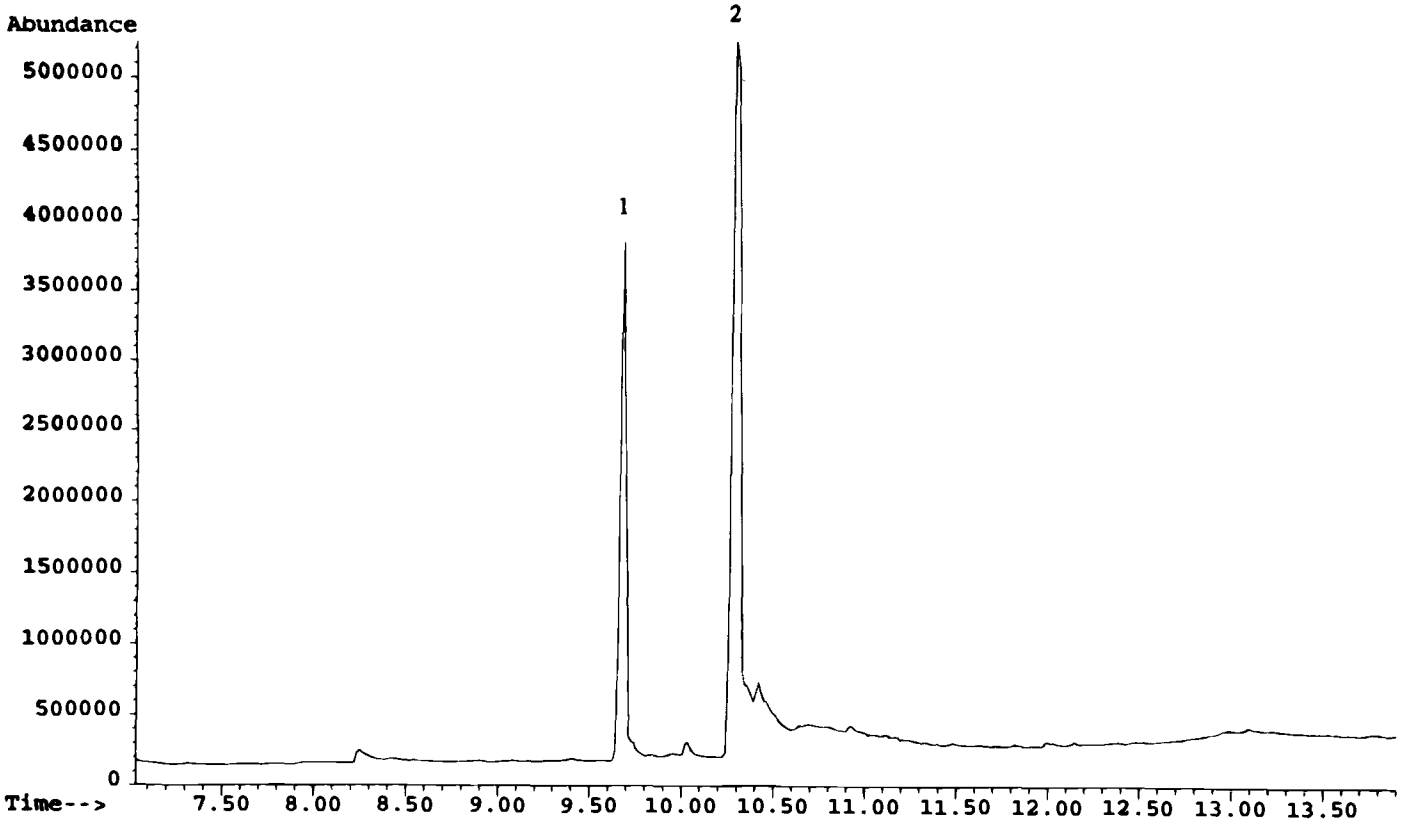

FIG. 1—*Total ion chromatogram of a typical urinary extract showing the separation between perfluorooctanoyl derivatives of N-propylamphetamine (internal standard) and phenmetrazine. The peak 1 is the derivatized internal standard and peak 2 is the derivatized phenmetrazine. The concentration of phenmetrazine was 20 μg/mL while the concentration of the internal standard was 10 μg/mL.*

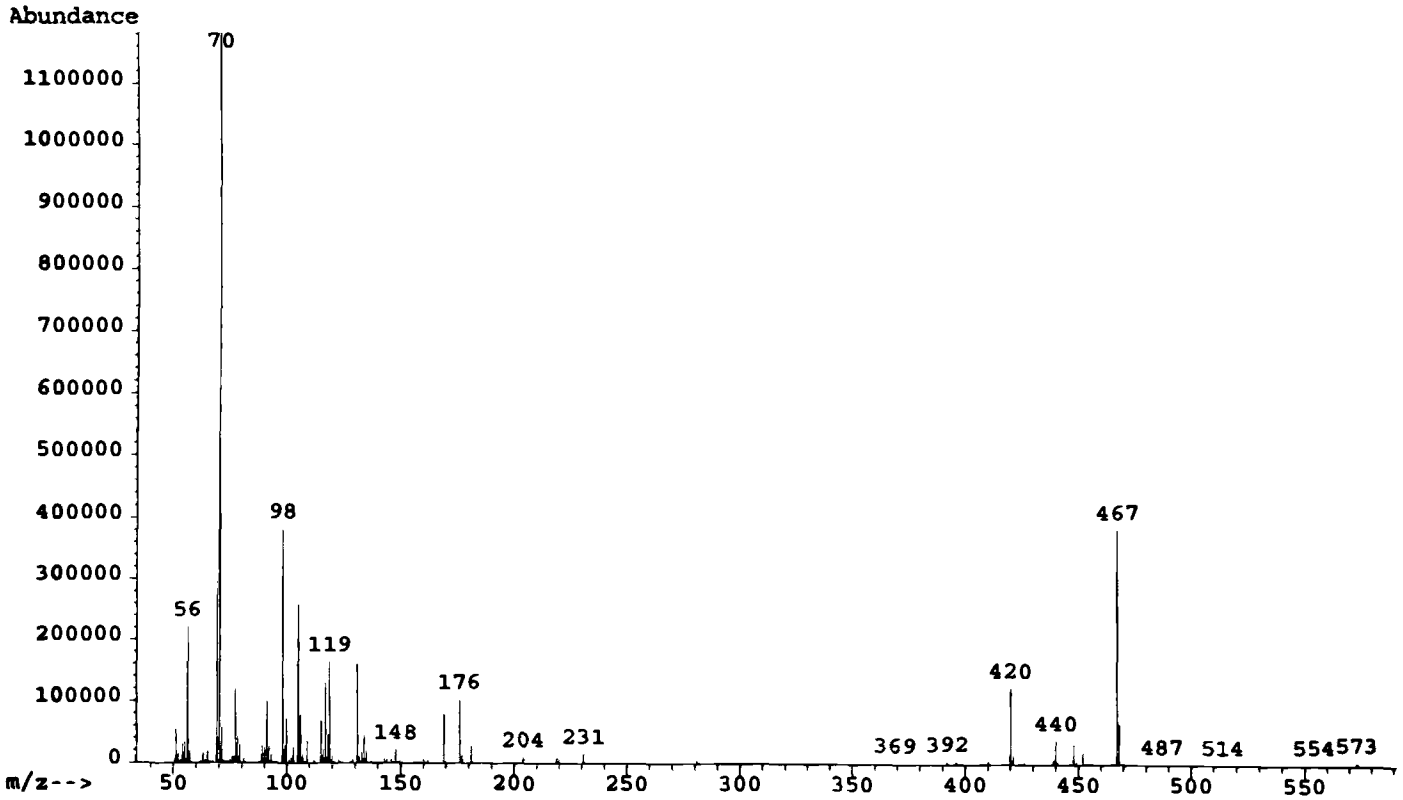

FIG. 2—*Mass spectrum of perfluorooctanoyl derivative of phenmetrazine using electron ionization.*

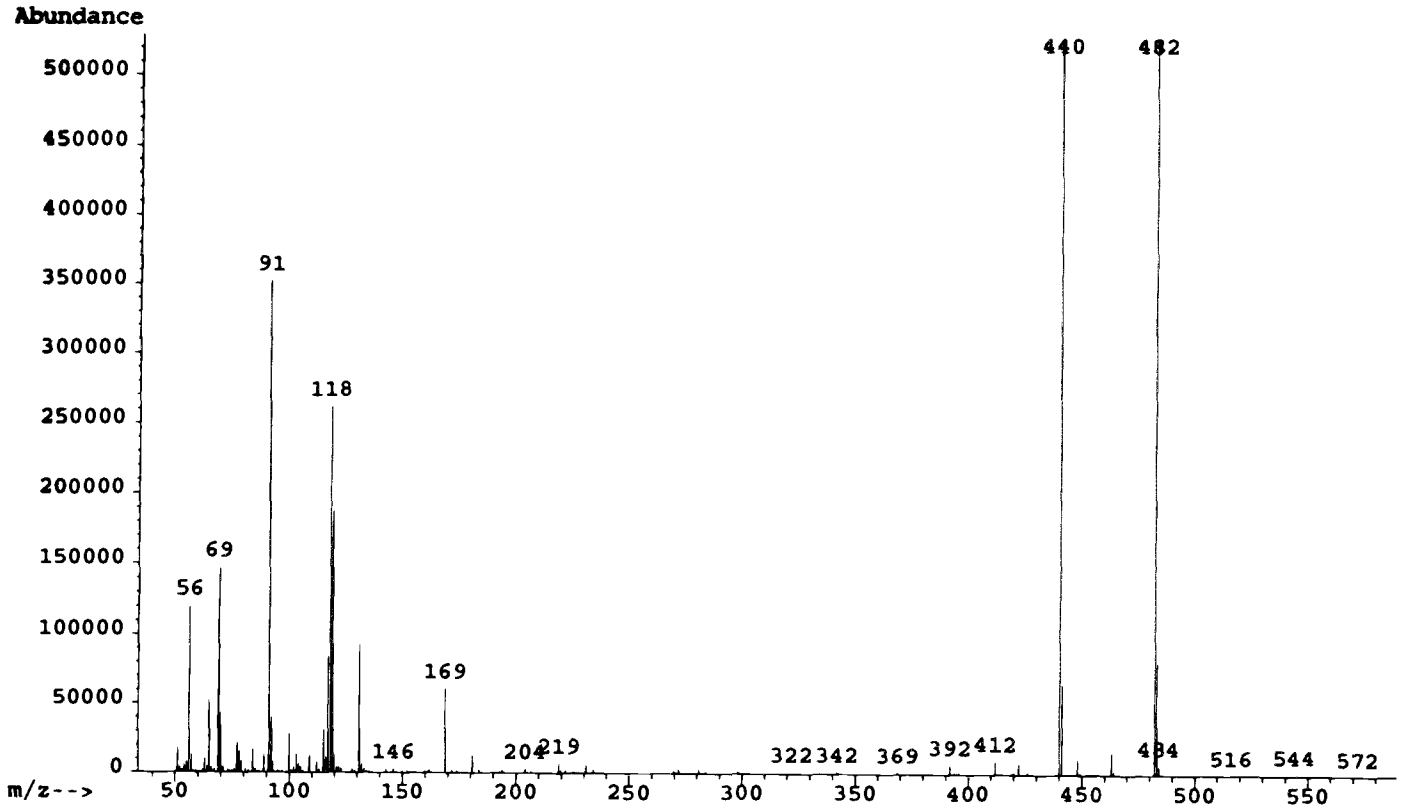

FIG. 3—*Mass spectrum of perfluorooctanoyl derivative of N-propylamphetamine (internal standard) using electron ionization.*

also observed strong peaks at m/z 118 (relative abundance: 50.0%) and 91 (relative abundance: 67.3%), thus aiding in unambiguous identification of the internal standard (Fig. 3).

The perfluorooctanoyl derivative of amphetamine showed a base peak at m/z 440 while the perfluorooctanoyl derivative of methamphetamine showed a base peak at m/z 454. The gas chromatographic retention times and major mass spectral ions of phenmetrazine, the internal standard, amphetamine, methamphetamine, and other amphetamine like compounds are listed in Table 1.

Precision Linearity, and Detection Limit

The within run and between run precisions were determined at two different concentration levels. The within run and between run precision at a urinary phenmetrazine concentration of 20 μg/mL were 1.7% ($\bar{X}$ = 20.20, SD = 0.35 μg/mL, n = 5) and 3.2% ($\bar{X}$ = 19.71, SD = 0.63 μg/mL, n = 5), respectively. However, the within run and between run precisions at a urinary phenmetrazine concentration of 1 μg/mL were 9.4% ($\bar{X}$ = 0.95, SD = 0.09 μg/mL, n = 5) and 10.8% ($\bar{X}$ = 1.11, SD = 0.12 μg/mL, n = 5), respectively. The assay was linear for urinary phenmetrazine concentration of 1 to 100 μg/mL. Using the X-axis as the target concentration and Y-axis as the observed concentration in the linearity study, we observed the following regression equation:

$$y = 0.96x + 0.04 \ (r = 0.99).$$

The detection limit was 0.5 μg/mL urinary phenmetrazine concentration, the same detection limit reported by Kronstrand et al. for determination of phenmetrazine in urine after derivatization with methyl chloroformate (6).

Application of the Assay

Because phenmetrazine is abused, a convenient GC/MS assay for unambiguous identification and quantitation of the drug is essential for both clinical and forensic toxicology laboratories.

TABLE 1—*Gas chromatographic retention time and mass-spectral characteristics of perfluorooctanoyl derivatives of phenmetrazine, N-propylamphetamine (internal standard), amphetamine, methamphetamine, and other amphetamine like compounds.*

Compound	Retention Time	Major ions, m/z, (Relative Abundance)		
Phenmetrazine	10.3	467 (35%)	98 (34%)	70 (100%)
N-Propylamphetamine	9.7	482 (100%)	440 (99%)	91 (76%)
Amphetamine	8.3	440 (100%)	118 (88%)	91 (46%)
Methamphetamine	9.2	454 (100%)	410 (21%)	118 (31%)
MDMA	11.1	454 (63%)	410 (32%)	162 (100%)
MDPA	11.4	482 (43%)	440 (69%)	162 (100%)
Phenylpropanolamine	8.0	423 (100%)	131 (91%)	104 (78%)
Phentermine	8.4	454 (100%)	132 (12%)	91 (24%)
Pseudoephedrine	10.5	454 (100%)	410 (15%)	131 (21%)

essential for both clinical and forensic toxicology laboratories. Several deaths were also reported from phenmetrazine overdoses and unambiguous structural identification of the agent is essential for medico-legal cases. Phenmetrazine at the concentration of 1 μg/mL did not cross-react with the amphetamine EMIT immunoassay. We observed cross reactivity with EMIT immunoassay at a urinary phenmetrazine concentration of 6.6 μg/mL and higher. Therefore, if abuse of phenmetrazine is suspected, an unambiguous confirmation by mass spectrometry is essential. The major advantage of the perfluorooctanoyl derivative over the carbamate derivative is lesser volatility of the perfluorooctanoyl derivative. Therefore, the assay is free from interferences by volatile components of the matrix. Moreover, we observed strong characteristic ions in the higher mass range in the mass spectrum of perfluorooctanoyl derivative of phenmetrazine thus aiding in unambiguous confirmation of the drug if present in urine. The broad linearity range of the assay should aid in the investigation of abuse of phenmetrazine or of death from a suspected overdose.

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