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TECHNICAL NOTE

TOXICOLOGY

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Rapid and Simple GC–MS Method for Determination of Psychotropic Phenylalkylamine Derivatives in Nails Using Micro-Pulverized Extraction*

ABSTRACT: A rapid and simple gas chromatography–mass spectrometry (GC–MS) method was developed and validated for the simultaneous detection and quantification of five psychotropic phenylalkylamines (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, and norketamine) in toenails. After external decontamination, nail clippings were mechanically pulverized with a bead mill and then incubated in methanol under ultrasonication at 50°C for 1 h. The resulting solutions were evaporated to dryness, derivatized, and analyzed by GC–MS. The intra- and inter-day precisions were within 10.7% and 13.9%, respectively. The intra- and inter-day accuracies were −4.2% to 5.0% and −2.4% to 8.4%, respectively. Limits of detection and quantification for each analyte were lower than 0.024 and 0.08 ng/mg, respectively. The recoveries were in the range of 80.6–87.5%. The results indicated that the proposed method is a simple, rapid, accurate, and precise for quantification of five phenylalkylamines in nails. The method was successfully applied to the simultaneous detection and quantification of phenylalkylamines in nail samples of possible drug abusers.

KEYWORDS: forensic science, nail analysis, phenylalkylamine, micro-pulverized extraction, high-speed centrifugation, gas chromatography–mass spectrometry

Drugs, biological substances, and trace elements are accumulated in nails. Nails may give a history of drug intake and abuse and therefore represent a unique matrix for forensic toxicology purposes like hair (1).

In recent years, drug testing in keratinized matrices of hair and nails has received considerable attention because of its advantages over conventional urine or blood tests (2). A primary limitation of urine and blood analyses is the relatively short retrospective time period for detecting drug use. The nail provides an easily accessible matrix that can be employed in drug testing to detect illicit drugs and postmortem detection of abused drugs. As the drug and metabolites are kept in a stable manner, the detection of the metabolites of drugs of abuse gives proof of drug ingestion.

For several decades, nail analysis has been used for detecting transition metals and drugs of abuse including amphetamine-type stimulants, cannabinoids, opiates, cocaine, phencyclidine, benzodiazepines, and methadone (3–12). However, isolating drugs from nail matrix is time consuming and laborious. Alkaline digestion is conventionally used for nail sample preparation, which causes a chemical destruction of keratinized matrices and the stability of analytes may be affected during the hydrolysis procedure (13). Thus, nail components are extensively hydrolyzed, and additional cleanup procedures are required to achieve effective removal of matrix interferences.

To overcome these difficulties with nail analysis, micro-pulverized extraction and subsequent high-speed centrifugation were applied for sample preparation in the study. Mechanical pulverization of nail, methanolic extraction, and subsequent purification steps could be useful in accomplishing rapid and easy sample preparation.

In this study, we describe the rapid and simple method for the detection of psychotropic phenylalkylamine derivatives in nails using micro-pulverized extraction for the first time. The use of mechanical pulverization and a high-speed centrifuge was introduced to reduce interference from the nail matrix and to enhance detection sensitivity of target analytes. The method was validated and evaluated for its feasibility and applicability with real samples of nails obtained from suspected drug abusers.

Materials and Methods

Chemicals

The reference compounds amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), and norketamine (NKT) were purchased from Cerilliant (Austin, TX) at a concentration of 1000 μg/mL in methanol, and methanolic solutions of the deuterated internal standards, AP-d_8, MA-d_{11}, MDA-d_5, MDMA-d_5, and

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NKT-d_4 at 100 μg/mL were also purchased from Cerilliant. Heptafluorobutyric anhydride (HFBA) was supplied from Acros Organics (Geel, Belgium). HPLC-grade methanol and ethyl acetate were supplied from J. T. Baker (Phillipsburg, NJ). The water was purified using a Direct-Q water purification system (Millipore, Bedford, MA); 1.5- and 2.0-mL safe-lock tubes were purchased from Eppendorf (Hamburg, Germany). Zirconia balls with a 3 mm diameter were purchased from Samhwa Ceramics (Seoul, Korea).

Preparation of Solutions

Working standard solutions (0.1, 1.0, 10.0 μg/mL) of AP, MA, MDA, MDMA, and NKT were prepared by appropriate dilution with methanol. Internal standards were prepared in methanol to give a combined working standard solution of 0.5 μg/mL for AP-d_8, MA-d_{11}, MDA-d_5 and MDMA-d_5, and 1.0 μg/mL for NKT-d_4. All of these solutions were stored at −20°C in the absence of light until used.

Nail Specimens

Drug-free toenails obtained from six volunteers including two laboratory employees were used to prepare the matrix for the control and calibration samples and to measure the growth rate of toenail. Toenail samples of possible drug abusers were obtained by cutting the excess overhang of the nail plate from the Narcotics Departments at the District Prosecutors' Offices. A total of four samples were collected from drug abusers including positive samples tested for MA use during a screening test of urine samples by gas chromatography–mass spectrometry (GC–MS). The length of the nail clippings was measured, and special treatments such as manicuring and artificial nail tips were noted.

Sample Preparation

Toenail sample was first washed with water (5 mL) and subsequently washed three times with methanol (5 mL). It was then air-dried, cut in segments of below 0.5 cm each, measured 20 mg of sample, and finally pulverized in a 2-mL safe-lock tube containing five Zirconia beads with a bead mill (Qiagen TissueLyser II; Retsch, Haan, Germany). Pulverization was performed at a frequency of 30 Hz for 10 min. The combined internal standard solution (50 μL, 0.5 μg/mL for AP-d_8, MA-d_{11}, MDA-d_5, and MDMA-d_5, and 1.0 μg/mL for NKT-d_4) and 1.2 mL of methanol were then added. The tube was capped and shaken up and down more than once. Then, the sample was extracted under ultrasonication in a water-bath equipped with thermostat at 50°C for 1 h. After the tube was centrifuged at 10,000 × g for 5 min, the supernatant was loaded into a new 1.5-mL safe-lock tube and centrifuged again at high speed (30,000 × g for 5 min). The upper phase (1 mL) was transferred to a test tube (12 × 100 mm) and concentrated to dryness under a nitrogen stream at 40°C and 30 kPa using a TurboVap evaporator (Caliper Life Sciences, Hopkinton, MA). It was then dried in a vacuum desiccator over silica gel for at least 10 min. Heptafluorobutyryl (HFB) derivatives of AP, MA, MDA, MDMA, and NKT were formed by reaction of the sample with 50 μL ethyl acetate and 50 μL HFBA in a dry heating block at 60°C for 30 min, followed by drying under a nitrogen stream. The residue was reconstituted with 50 μL of ethyl acetate. An aliquot (1 μL) of sample solution was injected into the GC–MS.

GC–MS Analysis

GC–MS analyses were performed with an Agilent Technologies 5975 inert mass spectrometer (Foster City, CA) equipped with a 6890N GC and 7683B automatic liquid sampler. Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Tech., MSD Chemstation D.02.00). Separation was achieved with a capillary column (DB-5MS, 30 m × 0.25 mm i.d., 0.25 μm; J&W Scientific, Folsom, CA) with helium as the carrier gas at a flow rate of 1.1 mL/min. The GC temperature program was as follows: Initial temperature was 90°C for 3.0 min, increased to 170°C at a rate of 15°C/min, held for 2.0 min, increased to 210°C at a rate of 25°C/min, held for 1.5 min, then increased to 230°C at a rate of 20°C/min, held for 0.5 min, finally increased to 300°C at a rate of 40°C/min, and held for 0.3 min. Splitless injection mode was used with a purge-on time of 0.1 min at flow rate of 16.5 mL/min. The injector and the GC interface temperatures were 260 and 280°C, respectively. The mass spectrometer was operated at 70 eV in the electron impact mode with selected ion monitoring (SIM) for quantification. Quantifier and qualifier ions were monitored in their respective groups for each compound. These compounds had different elution times that are listed with the elution order in Table 1.

Validation of the Analytical Method

The method was validated and tested according to protocol before the application to real samples (14). Selectivity, matrix effect, linearity, limits of detection (LOD), and limits of quantification (LOQ), precision and accuracy, and recovery were assayed for five phenylalkylamine derivatives in toenail.

TABLE 1—*Retention times, molecular weights, and ions monitored for gas chromatography–mass spectrometry analysis for heptafluorobutyryl (HFB) derivatives.*

Compound	Retention Time (Min)	Molecular Weight	Ions Monitored (m/z)		
			Quantifier Ions	Qualifier Ions	
AP-d_8-HFB	7.92	339	243	–	–
AP–HFB	7.95	331	118	240	91
MA-d_{11}-HFB	8.96	356	260	–	–
MA–HFB	9.02	345	254	118	210
MDA-d_5-HFB	11.54	380	167	–	–
MDA–HFB	11.57	375	162	135	375
MDMA-d_5-HFB	12.55	394	258	–	–
MDMA–HFB	12.58	389	254	162	210
NKT-d_4-HFB	13.12	424	388	–	–
NKT–HFB	13.14	420	384	356	340

AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine.

To evaluate selectivity, drug-free toenail samples were extracted and analyzed for peaks interfering with the detection of the analytes or the internal standards. Potential interference from phenylalkylamine derivatives was also investigated using the spiked sample at a concentration of 1.0 ng/mg of each analyte.

Calibration curves were constructed over the LOQ for all the analytes. Linear regression analysis was performed on the peak area ratios of analyte to internal standard versus analyte concentrations. The LOD and LOQ for each analyte were estimated in accordance with the baseline noise from drug-free nail extracts. The baseline noise was evaluated by recording the detector response over a period of about 10 times the peak width. The LOD was obtained as the concentration of a sample that provided a peak with a height three times the baseline noise level, and the LOQ was calculated as 10 times the baseline noise level.

Seven replicates at the three different quality control (QC) sample concentrations (0.3, 3.0, and 10.0 ng/mg) were added to drug-free samples and extracted as above for the determination of intra- and inter-day precision and accuracy. The inter-day precision and accuracy were determined for four independent experimental days ($n = 4$). To determine the precision, relative standard deviation (% RSD) was calculated for the replicate measurements. Expressed accuracy (% bias) as the relative error of the calculated concentrations is calculated by the degree of agreement between the measured and nominal concentrations of the fortified samples.

Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the reference compounds in the extract from drug-free samples prior to and after the extraction procedure. The recoveries were assessed by QC samples using eight replicates for each QC sample concentration.

Results and Discussion

Sample Preparation

To isolate drugs from the nail matrix, alkaline digestion is generally favored. Hydrolysis procedures at high temperature lead to the breakage of keratin proteins in the nail, which causes considerable interference and results in background noise in the mass spectrum. Thus, additional cleanup procedures are required to achieve effective removal of matrix interferences.

To overcome difficulties, micro-pulverized extraction and subsequent high-speed centrifugation were introduced for sample preparation in the study. Mechanical pulverization of nail, methanol extraction, and subsequent purification step by high-speed centrifuge was useful in accomplishing rapid and easy sample preparation. These sample preparation techniques allow obtaining cleaner extracts to a great extent compared to alkaline hydrolysis and subsequent liquid–liquid extraction (LLE) (Fig. 1).

Toenail Growth Rate

Toenail growth rate was measured using a vernier calipers based on specimens collected from two laboratory employees for 3 years. Individual toenail growth rate data and mean values are detailed in Table 2. The mean toenail growth rates of two subjects for 3 years were 1.64 and 1.66 mm/month (mean 1.65) while those of big toes ranged from 1.87 to 1.95 mm/month (mean ± SD, 1.92 ± 0.04). These growth rate data could be used to confirm the long-term administration of illicit drugs in toenails as segmental hair analysis was used to verify both their previous drug history and recent enforced abstinence.

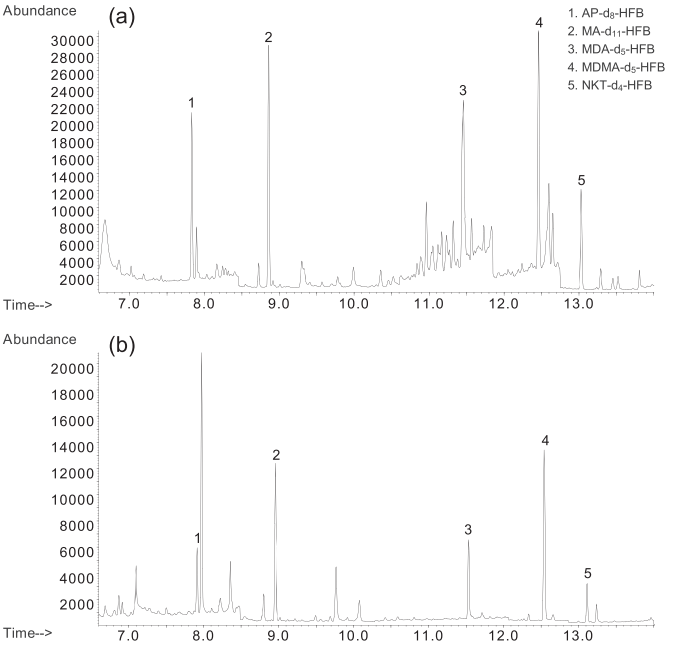

FIG. 1—*Comparison of representative chromatograms obtained from extracts of nail samples through (a) alkaline hydrolysis and liquid–liquid extraction and (b) micro-pulverized extraction and high-speed centrifugation. AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine.*

GC–MS Analysis

Derivatization in GC–MS analysis improves overall chromatographic selectivity and nontailing peak shapes, producing new compounds with altered polarity and volatility and forming distinctive mass spectral fragment ions. Acylating reagents such as trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), and HFBA are used for chemical derivatization of amphetamine-type stimulants. Derivatization with HFBA gave better selectivity and recovery for MA and AP compared with TFAA and PFPA (12). In the study, the optimum reaction condition for derivatization was 60°C and 30 min, which provided the best overall chromatographic selectivity and nontailing peak shapes.

Electron impact mass spectra, as obtained with the quadrupole mass spectrometer, of the derivatized analytes and corresponding internal standards are shown in Fig. 2. Under the conditions used for analysis (nominal electron energy 70 eV), characteristic ions of

TABLE 2—*Measurement of mean toenail growth rate of two subjects for 3 years (mm/month).*

	Subject 1	Subject 2	Mean Value
Big toe (right)	1.87	1.91	1.89
Big toe (left)	1.94	1.95	1.95
Index toe (right)	1.60	1.60	1.60
Index toe (left)	1.58	1.60	1.59
Middle toe (right)	1.63	1.74	1.68
Middle toe (left)	1.60	1.62	1.61
Fourth toe (right)	1.67	1.65	1.66
Fourth toe (left)	1.65	1.63	1.64
Little toe (right)	1.48	1.47	1.48
Little toe (left)	1.41	1.45	1.43
Standard deviation	0.16	0.16	0.16
Mean value	1.64	1.66	1.65

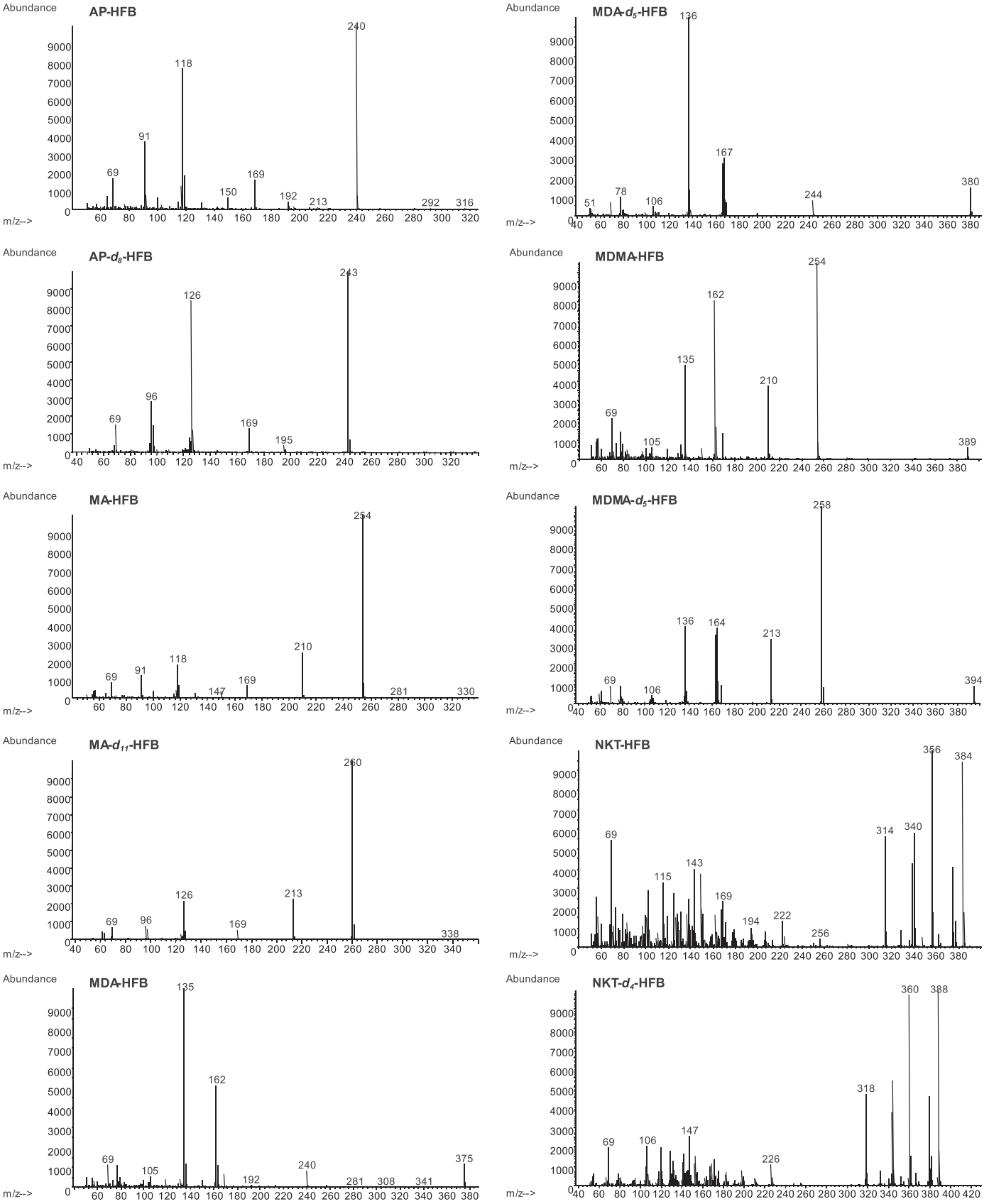

FIG. 2—*Electron impact mass spectra for heptafluorobutyryl (HFB) derivatives of the analytes. AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine.*

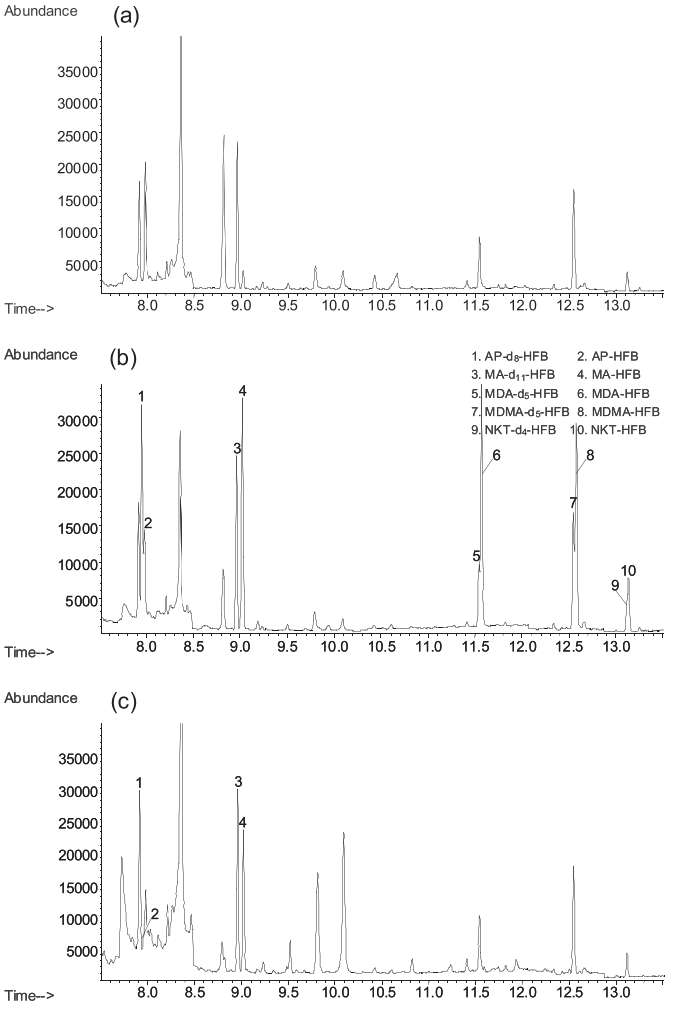

FIG. 3—*Gas chromatography–mass spectrometry merged selected ion chromatograms for heptafluorobutyryl (HFB) derivatives of the analytes and internal standards, including (a) drug-free toenail, (b) drug-spiked toenail at 1.0 ng/mg of each analyte, and (c) drug-user toenail sample. AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine.*

HFB derivatives of AP, MA, MDA, MDMA, and NKT are described in Table 1.

Evaluation of the Validation Data

The selectivity of the method was assessed by analyzing drug-free toenail, drug-spiked toenail, and drug-user toenail samples. Representative chromatograms obtained from GC–MS SIM mode are shown in Fig. 3. All analytes were well separated with good peak shapes. Figures 1–3 showed no interfering peaks from endogenous substances or co-extracted compounds.

Seven-point calibration curves for each analyte were established with three replicates at each concentration. The linear ranges were 0.05–15.0 ng/mg for AP, MDA, MDMA, and NKT, and 0.08–15.0 ng/mg for MA with the coefficients of determination ($r^2 \geq 0.9989$). LODs for each compound were lower than 0.024 ng/mg, based on the concentration of analyte corresponding to a signal intensity plus three standard deviations (SD) from the mean of eight replicates of drug-free hair. LOQs, defined as the concentration of analyte giving a signal equivalent to above 10 SD of blank signal, were lower than 0.08 ng/mg for the analytes (Table 3).

Analytical recovery, accuracy, and precision experiments were performed at three concentrations (low, middle, and high), over the calibration range. The intra-day ($n = 3$) and inter-day ($n = 4$) accuracy (% bias) and precision (% RSD) were assessed by analyzing seven QC samples spiked with the analytes at three different concentrations (0.3, 3.0, and 10.0 ng/mg). The intra-day and inter-day precisions were within 10.7% and 13.9%, respectively. The intra-day and inter-day accuracies were between −4.2% and 5.0% and −2.4% and 8.4%, respectively (Table 4). These results are satisfactory given the complexity of toenail matrix after micro-pulverized extraction. Analytical recoveries at three concentration levels in five replicates were 80.6–87.5%.

Application to Real Samples from Drug Abusers

The applicability of the method was examined using real toenail samples from suspected illicit drug abusers. A total of four toenail samples obtained from the Narcotics Departments at the District Prosecutors' Offices were analyzed for the analytes. Of the samples tested, only one toenail sample tested positive for MA and AP. MA was the most frequently detected compound, in association with its major metabolite AP. Figure 3*c* shows the representative chromatogram of an MA abuser's toenail sample.

The application of the described methodology allows the simultaneous detection and quantification of the mentioned compounds in toenail samples. The simultaneous detection and quantification of several kinds of abused drugs is advantageous to forensic toxicologists who are frequently confronted with limited sample size and detection of multiple drug use. Unfortunately, availability of toenail samples from drug abusers was limited for the present study because of the protection of the rights of suspects.

Conclusions

A simple and reliable GC–MS method was developed for determination of five phenylalkylamine derivatives in human toenail.

TABLE 3—*Calibration curve results, LOD, and LOQ for analytes.*

Analyte	Concentration Range (ng/mg)	Slope	y-Intercept	Linearity (r^2)*	LOD (ng/mg)†	LOQ (ng/mg)‡
AP	0.05–15.0	0.7561	0.0303	0.9991	0.015	0.05
MA	0.08–15.0	0.8450	0.0617	0.9994	0.024	0.08
MDA	0.05–15.0	1.3932	0.0220	0.9994	0.014	0.05
MDMA	0.05–15.0	0.7938	0.0183	0.9995	0.012	0.05
NKT	0.05–15.0	0.8471	0.0527	0.9989	0.014	0.05

AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine.

*Linearity is described by the correlation coefficient for the calibration curve.

†Limit of detection (LOD) is based on the concentration corresponding to a signal plus 3 and 10 standard deviations from the mean of eight replicates of drug-free toenail.

‡Limit of quantification (LOQ) is based on the concentration corresponding to a signal plus 3 and 10 standard deviations from the mean of eight replicates of drug-free toenail.

TABLE 4—*Analytical recovery, intra-day, inter-day, inter-person precision and accuracy.*

Analyte	Nominal Concentration (ng/mg)	Recovery (% Mean ± SD)	Intra-Day (n = 3)		Inter-Day (n = 4)	
			Precision (% RSD)*	Accuracy (% Bias)†	Precision (% RSD)	Accuracy (% Bias)
AP	0.3	84.4 ± 3.9	10.7	1.0	13.9	3.8
	3.0	86.5 ± 2.4	4.1	2.1	3.3	3.7
	10.0	86.9 ± 1.0	2.9	−0.3	5.5	1.9
MA	0.3	81.5 ± 5.2	6.8	−1.0	13.5	3.7
	3.0	81.6 ± 3.0	4.2	0.4	3.0	2.7
	10.0	86.0 ± 4.1	2.5	−1.4	6.6	−0.2
MDA	0.3	81.3 ± 1.2	8.5	4.9	6.5	6.3
	3.0	83.6 ± 1.6	4.5	2.4	1.1	3.9
	10.0	85.7 ± 1.9	3.3	−0.5	4.3	−2.2
MDMA	0.3	80.6 ± 1.1	4.9	1.7	9.7	5.0
	3.0	82.6 ± 2.9	4.8	2.0	1.8	3.5
	10.0	85.7 ± 1.6	2.7	−0.5	3.7	−2.4
NKT	0.3	82.3 ± 1.0	5.2	−4.2	9.5	8.4
	3.0	84.0 ± 3.0	5.4	5.0	2.4	5.6
	15.0	87.5 ± 1.2	3.3	−0.1	4.2	−0.5

AP, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NKT, norketamine; RSD, relative standard deviation.

*Expressed as the coefficient of variance of the peak area ratios of analyte/internal standard.

†Calculated as [(mean calculated concentration−nominal concentration)/nominal concentration] × 100.

The method includes pulverization, methanol extraction, and HFB derivatization of the analytes. Toenail pulverization using a bead mill allows obtaining more clean extracts to a great extent compared to alkaline hydrolysis and subsequent LLE. The method also shows efficient micro-pulverized extraction, better detection sensitivity, and decreased amount of sample preparation time for analysis. The proposed method has been validated and effectively applied to real toenail samples from drug users.

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