Determination of Amphetamine, Methamphetamine, and Hydroxyamphetamine Derivatives in Urine by Gas Chromatography–Mass Spectrometry and Its Relation to CYP2D6 Phenotype of Drug Users

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Abstract

Amphetamine, a CYP2D6 substrate, is widely used by truck drivers, and the extent to which different people metabolize the drug has only been determined in an isolated or reduced number of samples. A gas chromatography–mass spectrometry method is implemented to simultaneously determine amphetamine, methamphetamine, and hydroxyamphetamine in the urine of drug users. This method is a useful contribution to a well-established field. The main improvements are the use of liquid–liquid extraction, the trapping of the amphetamines as their hydrochloride salt, as a solution to the volatility of these analytes, and its application to assess the CYP2D6 metabolic phenotype of amphetamine users, which is innovative. Calibration curves ranged from 125 to 1000 ng/mL and had an r^2 greater than 0.99. The validation data (precision, accuracy, and recovery) shows the reproducibility and selectiveness of the method. The method is applied to determine the metabolic ratio (MR) in 121 urine specimens of federal highway drivers who underwent random mandatory roadside testing for drugs. The statistical analysis of the MR shows the presence of three different groups, which according to the established groups for CYP2D6 and the amount of the drug metabolized, are classified into extensive metabolizers (EM), intermediate metabolizers (IM), and poor metabolizers (PM). The biological consequences of these differences in amphetamine metabolism, such as impaired driving, a risk to develop Parkinson's disease, or an addiction, need to be further studied.

Introduction

Amphetamines are sympathomimetic amines that include a number of therapeutic agents and illicit drugs. The abuse of amphetamines is a serious problem among youths attending recreational settings and truck drivers.

The hydroxylation of the aromatic ring, or its substituents, is a major route of the metabolism of amphetamines (1). Hydroxylation at either the para or meta position is favored by CYP2D6 because these positions are 5 to 7 angstroms from the basic nitrogen of the amphetamines (2). The amphetamines that are ring-hydroxylated by CYP2D6 are amphetamine (3) and methamphetamine (4).

The CYP2D6 gene is located in the long arm of chromosome 22. Its product is involved in the oxidative metabolism of a variety of drugs, including antiarrhythmics, antidepressants, narcoleptics, beta-blockers, opioids, and amphetamines (5). Enzyme activity is known to vary between individuals because of differences in genetic polymorphisms (6). A deficiency in the hydroxylation of amphetamine was one of the observations that led to the discovery of CYP2D6 polymorphism (7). In a study published in 1970 by Dring et al. (8), *p*-hydroxyamphetamine was found in the urine of two out of three individuals who received a single oral administration of the radiolabeled enantiomers of amphetamine. In 1986, the third subject was shown to have a CYP2D6 deficiency (9). CYP2D6 genotyping studies have revealed a large number of alleles (6–10). Individuals with the usual CYP2D6 activity are called extensive metabolizers (EM), and those with lower levels of activity are known as poor metabolizers (PM). Individuals exhibiting a rate of metabolism between that of EM and PM are identified as intermediate metabolizers (IM). There is a high variation between ethnic groups. PMs occur in 5–8% of whites and 2–10% of Africans and Asians (11). In the European population, 5–10% is homozygous for inactivating mutations and lacks the respective enzyme activity (7), though Ethiopians carry duplicate or even multicopy active CYP2D6 that result in an ultra-rapid enzyme and have hence been called ultrametabolizers (UM) (12).

In PM, the elimination of drugs is reduced, and repeated in-

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gestion can lead to accumulation and toxicity. In an attempt to apply this knowledge to clinical practice, genotyping and phenotyping are used to classify the type of metabolizer, and the results are usually concordant (5–13). In particular, phenotyping is advantageous because it provides direct information on the actual level of enzyme activity. To determine the phenotype of CYP2D6, the metabolic ratio (MR) is calculated by dividing the concentration of the parent compound in urine by the concentration of the metabolite. The CYP2D6 activity is substrate-dependent, and for this reason, each drug must be evaluated (14).

In the case of amphetamines, ethical and legal implications make the evaluation of the MR in healthy volunteers difficult. For this purpose, samples of drug users obtained during a mandatory random roadside testing of drugs, carried out by the Mexican Secretariat of Communications and Transport, represented a good alternative for its evaluation.

A variety of analytical methods to measure concentrations of amphetamine and methamphetamine in biological fluids are in use (15–17), but the presence of the hydroxylated metabolite in urine has only been reported in a reduced number of individuals (18,19). In this study, a selective and reproducible method for the simultaneous determination of two amphetamines and the hydroxylated metabolite in urine samples was implemented to assess the CYP2D6 metabolic phenotype of amphetamine abusers.

Experimental

Chemicals

Amphetamine, amphetamine-d_5, methamphetamine, and methamphetamine-d_5 were purchased from Cerilliant (Austin, TX) as 1 mg/mL solution in methanol. Hydroxyamphetamine was supplied by the Laboratory of Toxicology of the Secretariat of Communications and Transport. The bis(trimethylsilil)-trifluoroacetamide (BSTFA) and trimethyl chlorosilane (TMCS) were purchased from Sigma (St. Louis, MO). Methanol, dichloromethane, acetone, and methanol were chromatographic grade from Merck (Darmstadt, Germany). Sodium hydroxide and hydrochloric acid (HCl) were of analytical reagent grade and bought from Merck.

Analytical procedure

Stock standards for amphetamine, methamphetamine, hydroxyamphetamine, and deuteriated standards were prepared, sealed, and refrigerated at 4°C until used. Blank urine spiked with amphetamines or urine samples were extracted as follows: 2 mL from urine obtained was pipetted into 10 tubes and 25 µL internal deuterium standard solution (final concentration = 500 ng/mL), 0.5 mL 1N NaOH, and 3 mL dichloromethane were added. The tubes were capped, shaken, and centrifuged at a low speed for 5 min, and the upper aqueous layer was aspirated to waste. The organic layer was transferred to a new conical vial, into which 0.1 mL methanol/HCl (9:1) was added. The conical vial was evaporated to dryness under nitrogen. After evaporation, the dried extracts were derivatized by adding 40 µL BSTFA/TMCS (95:5), mixing, and incubating for 30 min at 60°C. The mixture (1 µL) was injected into a gas chromatograph–mass spectrometer (GC–MS).

Specimens

Urine samples (121) were obtained from approximately 1000 federal highway truck drivers (18 to 65 years old) who underwent mandatory random roadside testing for drugs. The drivers were asked to sign a written informed consent. A medical officer interviewed the individuals in order to document their therapeutic drug use. The specimens were taken following the procedures and guidelines for drug testing at the workplace of the Mexican Secretariat of Communication and Transport. Samples were screened by immunoassay analysis according to the manufacturer's instruction with EMIT Dade Behring for analyzing five families of drugs and alcohol (cocaine, Δ^9-tetrahydrocannabinol, opiates, phencyclidine, and amphetamines). Only positive samples for amphetamines were used for this study. Samples positive for alcohol or the other drugs, including those from the subjects reported to be exposed to a pharmacologic treatment, were excluded. The samples were coded to avoid the identification of the individual. Blank urine samples were obtained from the laboratory staff.

Apparatus

Analysis was performed with a model 5970 electron impact quadrupole MS interfaced with a 6890 GC (Agilent technologies). The GC was equipped with a 30-m HP-5 fused-silica capillary column (0.25-mm i.d. and 0.32-µm film thickness). The carrier gas, helium, had a flow of 1.0 mL/min. The operation mode was splitless. The injection port and transfer zone temperatures were maintained at 250°C. One microliter sample volume was injected. The initial oven temperature was set at 60°C for 1 min, then it was ramped at 10°C/min to 150°C and held for 8 min. The sampling analysis time was 10 min. A mass selective detector was run in scan mode (mass

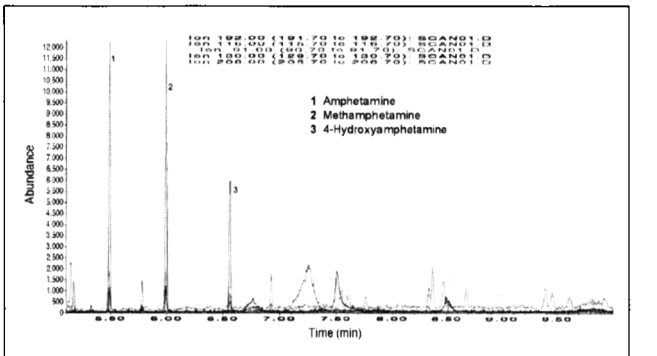

Figure 1. GC–MS-SIM chromatogram of amphetamine (conc. 250 ng/mL, peak 1), methamphetamine (250 ng/mL, peak 2), and 4-hydroxyamphetamine (125 ng/mL, peak 3). Sampling analysis time was 10 min. The mass selective detector was run in scan mode (mass range 50–400) to establish a fragmentation pattern of a structural diagnosis, and the final methodology was run in SIM using the masses given in Table I.

range 50–400) to establish a fragmentation pattern of structural diagnosis, and the final methodology was run in single-ion monitoring (SIM).

Results and Discussion

Optimization of the analytical procedure

The method implemented to determine the amphetamine, methamphetamine, and 4-hydroxyamphetamine in urine is a useful contribution to a well-established field. This analytical method is distinguished from other amphetamine methods because of *(i)* the use of one liquid–liquid extraction carried out with conventional solvents, which diminished the cost of the analytical procedure and was easy to perform; *(ii)* the trapping of the amphetamines as their HCl salt is an appealing solution to the problem of the volatility of these analytes, and this step facilitates the solvent evaporation at 50°C with a minimum loss of amphetamines; *(iii)* the use of deuterated internal standards (IS) provides the calibration of the method; *(iv)* validation and recovery studies for the extraction procedure proved that one extraction with dichloromethane was sufficient; and *(v)* results were improved by the use of a derivatization mixture, which had been suggested previously to avoid the adsorption and interaction of free amphetamines, with the column increasing the sensitivity and reproducibility of the analytical procedure (20).

An example of a chromatogram of a simultaneous detection of amphetamine, methamphetamine, and 4-hydroxyamphetamine is shown in Figure 1. The peaks of the compounds appear well resolved, confirming that the chromatographic conditions used are appropriate for the separation of all these amines with derivatization. MS were obtained and are shown in Figure 2. The retention times and the ions of structural diagnostic importance are exhibited in Table I.

Validation of the method

For the validation of the method, the data of calibration curves, correlation, precision, and accuracy of the method were calculated (21). An analysis of blank samples (n = 10) and samples spiked with amphetamines proved that there is not interference from the matrix in the determination. To validate, five calibration curves (n = 9 standards, two injections per standard) were evaluated in the range 125–1000 ng/mL, which were analyzed according to the procedure described in the Analytical Procedure section, showing to be linear with r^2 = 0.9960 for amphetamine, r^2 = 0.9981 for methamphetamine, and r^2 = 0.9972 for 4-hydroxyamphetamine. The quantification was achieved using peak-area ratios of the amphetamines to IS, followed by the least square regression. Validation results [precision, accuracy, and limits of detection (LOD) and quantitation (LOQ)] of the methodology are presented in Tables II and III. The LOD was calculated analyzing a series of samples containing increasingly lower concentrations of the analyte, where LOD was the lowest concentration at which the results satisfied two acceptance criteria: *(i)* the determination of the MS and *(ii)* the height peak must be up of the mean blank value plus three standard deviations (SDs). The LOQ was defined as the concentration at which all acceptance criteria were met, and the quantitative value was within ± 20% of the target concentration.

In order to calculate the analytical recovery, relative areas

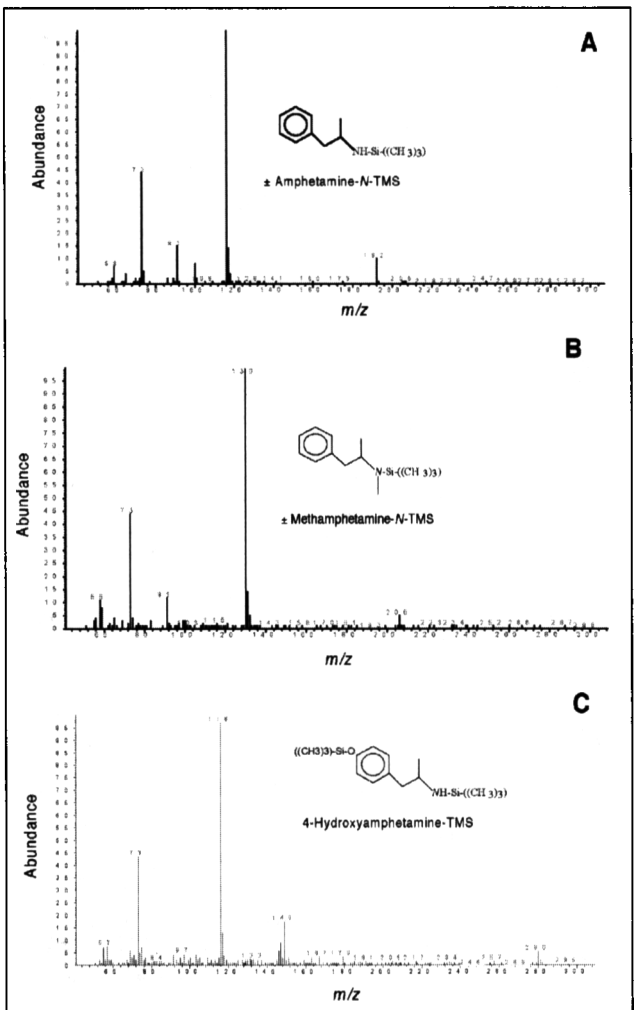

Figure 2. MS for amphetamine (A), methamphetamine (B), and 4-hydroxyamphetamine (C) in scan mode.

Table I. Detection of Amphetamine, Methamphetamine, and 4-Hydroxyamphetamine in Urine Samples by GC–MS

Compound	t_R* (min)	Ion m/z (Relative Abundance)†
Amphetamine	5.46	$\underline{116}_{(100)}$, $91_{(20)}$, $192_{(15)}$
Methamphetamine	5.99	$\underline{130}_{(100)}$, $91_{(15)}$, $206_{(11)}$
4-Hydroxyamphetamine	6.66	$\underline{116}_{(100)}$, $280_{(12)}$
Amphetamine-d_5 (IS)	5.44	$\underline{120}_{(100)}$, $92_{(19)}$, $197_{(13)}$
Methamphetamine-d_5 (IS)	5.97	$\underline{134}_{(100)}$, $92_{(14)}$, $211_{(12)}$

* t_R = retention time.
† Quantifier ions are underlined.

obtained from fortified urine samples (n = 10) with amphetamines were analyzed according to the Analytical Procedure section, and they were compared with relative areas obtained from amphetamine standard solutions, in which only evaporation and derivatization were carried out. The analytical recovery ranged from 61% to 82% for amphetamine, 60% to 80% for methamphetamine, and 64% to 85% for 4-hydroxyamphetamine. These results were obtained with only one extraction of dichloromethane, which showed to be sufficient.

Application of the method

Amphetamine is the most commonly consumed drug by Mexican truck drivers, in contrast with other laboral sectors and countries where methamphetamine is the main problem (22–23). Urine samples (121) of individuals positive for amphetamine in the immunoassay were evaluated using GC–MS during this study. Out of the tested subjects, 109 had amphetamine (90%) and 12 had methamphetamine (10%). The metabolite 4-hydroxyamphetamine was quantified in the amphetamine positive group. The ranges of drug

Table II. Validation Results: Intra- and Interassay Precision and Accuracy of the Assay

	Intra-assay				Interassay			
	Observed value				Observed value			
Theoretical Value (ng/mL)	Mean (ng/mL)	SD	RSD (%)	Accuracy (%)	Mean (ng/mL)	SD	RSD (%)	Accuracy (%)
Amphetamine								
125	134.33	10.68	7.95	107.46	142.10	13.39	9.42	113.68
200	207.97	13.56	6.52	103.99	218.10	17.51	8.03	109.05
250	247.13	8.31	3.36	98.85	235.90	13.26	5.62	94.36
375	382.60	11.79	3.08	102.03	377.70	16.66	4.41	100.72
500	507.47	7.15	1.41	101.49	495.10	13.07	2.64	99.02
625	633.00	28.05	4.43	101.28	631.20	19.19	3.04	100.99
750	736.60	17.19	2.33	98.21	739.80	17.61	2.38	98.64
875	886.27	25.91	2.92	101.29	868.60	27.88	3.21	99.27
1000	990.63	39.38	3.98	99.06	984.80	44.51	4.52	98.48
Methamphetamine								
125	117.83	9.09	7.71	94.26	122.30	10.76	8.80	97.84
200	187.70	13.36	7.12	93.85	197.82	13.25	6.70	98.91
250	254.03	12.11	4.77	101.61	247.78	12.96	5.23	99.11
375	363.13	8.32	2.29	96.83	371.84	17.22	4.63	99.16
500	496.70	9.9	1.99	99.34	497.93	13.49	2.71	99.59
625	622.90	11.22	1.80	99.66	627.63	15.57	2.48	100.42
750	735.33	16.78	2.28	98.04	736.04	26.64	3.62	98.14
875	865.50	25.65	2.96	98.91	863.92	33.17	3.84	98.73
1000	1013.20	22.04	2.18	101.32	1009.61	33.52	3.32	100.96
4-Hydroxyamphetamine								
125	117.13	10.4	8.88	93.70	120.92	11.79	9.75	96.74
200	195.07	11.97	6.14	97.54	187.64	13.40	7.14	93.82
250	256.80	14.11	5.49	102.72	255.71	10.28	4.02	102.28
375	365.47	9.68	2.65	97.46	363.13	8.39	2.31	96.83
500	502.37	9.51	1.89	100.47	496.70	12.42	2.50	99.34
625	628.97	11.85	1.88	100.64	622.89	16.38	2.63	99.66
750	740.63	15.72	2.12	98.75	735.30	21.69	2.95	98.04
875	871.23	29.63	3.40	99.57	865.57	35.06	4.05	98.92
1000	1019.07	56.32	5.53	101.91	10006.50	41.97	4.17	100.65

	Total average							
	Intra-assay				Interassay			
	RSD (%)	Range	Accuracy (%)	Range	RSD (%)	Range	Accuracy (%)	Range
Amphetamine	4.0	(1.41–7.95)	101.5	(98.2–107.5)	4.8	(2.4–9.4)	101.6	(94.4–113.7)
Methamphetamine	3.7	(1.8–7.7)	98.2	(93.9–101.6)	4.6	(2.5–8.8)	99.2	(97.8–100.9)
4-Hydroxyamphetamine	4.2	(1.9–8.9)	99.2	(93.7–102.7)	4.4	(2.3–9.8)	98.5	(93.8–102.3)

concentrations and their distribution are shown in Table IV.

The MR was calculated (amphetamine concentration/4-hydroxyamphetamine concentration). The cumulative and frequency distributions of these MRs are shown in Figures 3A and 3B, respectively. Figure 3A shows a trimodal behavior with the presence of three different slopes of MR range values (0.95–13.11, 15.22–24.96, and 28.57–100), which showed to be statistically different by the nonparametric Kruskall-Wallis test and also after logarithmic transformation by the ANOVA test ($p < 0.0001$). The classification of our subjects into EM, IM, and PM, established for CYP2D6 (5,24,25) is shown in Figure 3B. Only five individuals behaved as PM, showing very low amounts or none at all of the 4-hydroxy metabolite (MR values 28.57–100), and 90 individuals behaved as EM, showing the highest amounts of the metabolite (MR values ranged from 0.95 to 13.11). Those subjects with an IM performance (14) showed intermediate values of the metabolite (MR values ranged from 15.22 to 24.96).

The functionality of CYP2D6 has relevant implications for amphetamine users because for other drugs PM has been shown to have a higher risk of overdose toxicity than EM (11,26,27), and it might have important effects in relation to safe driving. The differences in metabolism might influence the risk of a driver having a crash because of the impairment of driving abilities, and this needs to be evaluated.

It is noteworthy to mention that a higher frequency of PM has been observed in patients with Parkinson disease, suggesting a role of CYP2D6 in the pathogenesis of this neurodegenerative disorder (28–30). Also, an increase of larynx and lung cancer has been found in UM smokers, indicating a role of CYP2D6 metabolic differences in cancer risk (31).

Table III. LOD and LOQ

Compound	LOD (ng/mL)	LOQ (ng/mL)
Amphetamine	10	30
Methamphetamine	10	30
4-Hydroxyamphetamine	15	50

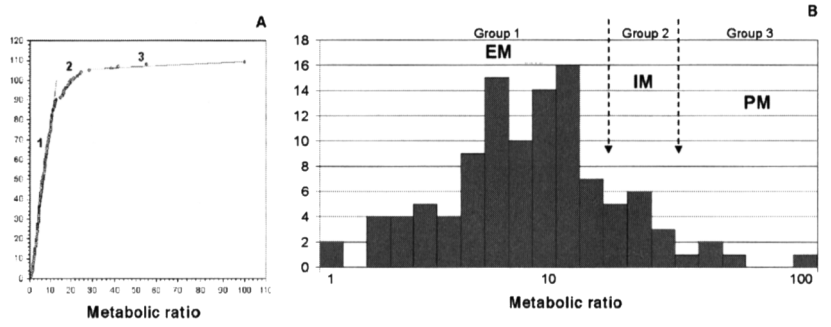

Figure 3. Distribution plots of MRs (amphetamine/4-hydroxyamphetamine) obtained from the concentrations determined on standard curves obtained from GC–MS analysis. Plot of cumulative number of individuals versus the MR, which shows three statistical different slopes of MR range values (0.95–13.11, 15.22–24.96, and 28.57–100) (A). Frequency distribution plot of amphetamine MR in drug users and their classification in three kinds of metabolizers: EM is MR = 0.95–13.11; IM is MR = 15.22–24.96; and PM is MR = 28.57–100 (B).

Conclusions

The implemented method allows the simultaneous analysis of amphetamine, methamphetamine, and 4-hydroxyamphetamine. The data obtained illustrate the application of a rapid, selective, and reproducible method for the assessment of individual metabolic differences because of a variation in the CYP2D6 phenotype. A deeper understanding of the individual differences in the metabolism of amphetamines could provide insight into the causes of abuse and addiction. Further studies are needed to understand the health and social consequences of the observed differences in the amphetamine metabolism.

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Table IV. Results of Urine Analysis of 121 Subjects (100%): 109 Amphetamine Positives (90%) and 12 Methamphetamine Positives (10%)

Amphetamine in Urine Range (ng/mL)	Number of Individuals	Methamphetamine in Urine Range (ng/mL)	Number of Individuals	4-Hydroxyamphetamine in Urine Range (ng/mL)	Number of Individuals
1000 to 5000	19	1000 to 5000	1	0 to 500	26
5000 to 10,000	44	5000 to 10,000	4	500 to 1000	25
10,000 to 20,000	33	10,000 to 20,000	4	1000 to 2000	30
20,000 to 50,000	12	20,000 to 50,000	2	2000 to 5000	18
> 50,000	1	> 50,000	1	> 5000	10
Total	109	Total	12	Total	109

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