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Article

Determination of Amphetamine, Amfepramone and Fenproporex in Urine Samples by HPLC-DAD: Application to a Population of Brazilian Truck Drivers

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Commercially available immunoassay tests are designed to detect the presence of amphetamine/methamphetamine or methylenodioxyamphetamines. However, it is known that Brazilian truck drivers also report the use of other illicit amphetamines, such as amfepramone and fenproporex. Thus, a method was developed and validated in order to quantify amphetamine-type stimulants (amphetamine, fenproporex and amfepramone) in urine by high performance liquid chromatography with diode array detection (HPLC-DAD). Prior to this, a liquid-liquid extraction (LLE) with diethyl ether was performed in order to extract the analytes. The limit of detection was 150 ng mL^{-1}. The method showed to be precise (relative standard deviation, RSD < 15%) and the recovery values for the three analytes were greater than 50%. The linearity ranged from 150 to 1000 ng mL^{-1} ($r^2 > 0.99$). Urine samples randomly collected from 385 truck drivers in Brazilian roads were submitted to the developed method. Nine samples were tested positive for amphetamine and one was tested positive for fenproporex and amphetamine.

Keywords: amphetamine, fenproporex, amfepramone, HPLC-DAD, truck drivers

Introduction

Freight transportation in Brazil is carried out predominantly on highways, which is responsible for more than 60% of all loads transported in the country.[1] Another fact that requires attention is the number of accidents involving lorries: in 2010, 88,963 cases were registered by the Brazilian Department of Federal Highway Police in federal roads.[2]

Certainly, traffic accidents are multi-causal phenomena; however the use of psychoactive substances is a contributing factor for their occurrence.[3-5] Indeed, Brazilian researches, which studied the consumption of these substances among truck drivers, through self-report or toxicological analysis, have detected mainly cannabinoids, cocaine and amphetamines, which are the focus of this study.[6-11]

Amphetamine-type stimulants (ATS) comprise a group of substances, mostly synthetic in origin, that are structurally derived from β-phenethylamine. ATS stimulate the central nervous system (CNS) and the most abused compounds that belong to this class are amphetamine itself (AMP), methamphetamine (MAP), 3,4-methylenedioxymethamphetamine (ecstasy or MDMA) and 3,4-methylenedioxyamphetamine (MDA).[12,13] Therapeutically, some of them can be used for the treatment of narcolepsy, attention deficit hyperactivity disorder and obesity. On the other hand, these substances are frequently abused due to their intense stimulant and psychedelic effects.[13] ATS are illegally produced in a variety of preparations (powder, tablets or capsules) and they may be injected, ingested orally, snorted or smoked.[12] World seizures of ATS have reached new highs: 123 tons in 2011 compared with 74 tons in 2010, a 66% rise, mainly due to surging methamphetamine seizures.[14]

In those epidemiological studies mentioned above, toxicological analyses were based on immunoassays screening tests followed by mass spectrometry methods to confirm positive results.[6,9,10] The commercially available immunoassay tests are designed to detect the presence of amphetamine/methamphetamine or methylenodioxyamphetamines. In recent studies conducted

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by our research group, Brazilian truck drivers report the use of fenproporex (FEN) and amfepramone (AMF), which also belong to the ATS class (Figure 1).[9,10] However, these substances are not effectively detected by common immunoassays, which could generate false-negative results and underestimate the number of positive cases.[15,16]

Liquid-liquid extraction (LLE) or solid-phase extraction (SPE) are both appropriate procedures for extracting drugs from urine, which do not usually require pretreatment prior to extraction.[17] Urine is the most commonly used biological matrix for testing of drugs of abuse. It is easy to collect (not invasive as blood) and to manipulate, and after a single dose of most types of drugs, the parent drug or a metabolite can be detected in urine for few days.[18]

The aim of the present study was to develop a method for the determination of amphetamine-type stimulants (AMP, FEN and AMF; Figure 1) in human urine samples by using LLE and high performance liquid chromatography with diode array detection (HPLC-DAD). The validated method was successfully applied to samples collected from truck drivers who were travelling through three different Brazilian highways in 2013. Positive results from the HPLC-DAD method were confirmed by gas chromatography-mass spectrometry (GC-MS).

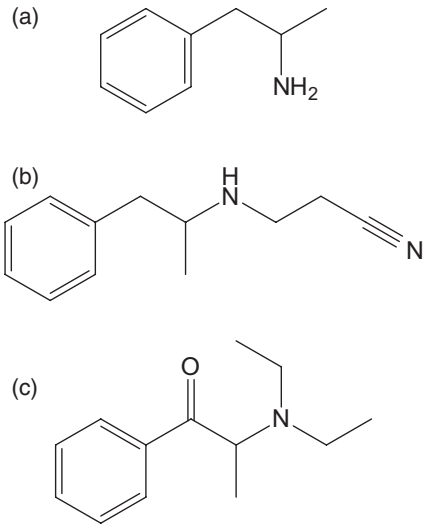

Figure 1. Chemical structures of the amphetamine-type stimulants: amphetamine (a); fenproporex (b) and amfepramone (c).

Experimental

Materials

Reagents and standards of reference

Amphetamine (1-phenylpropan-2-amine), fenproporex (3-(1-phenylpropan-2-ylamino)-propanenitrile), amfepramone (2-(diethylamino)-1-phenyl-1-propanone) and chlorprenaline, CLP (2-chloro-α-[[(1-methylethyl)-amino]-methyl]-benzenemethanol) (internal standard) solutions (1.0 mg mL^{-1}) in methanol were purchased from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Acetonitrile HPLC grade, methanol HPLC grade, sodium chloride, diethyl ether, anhydrous sodium sulfate, phosphoric acid solution, potassium hydroxide and hydrochloric acid were purchased from Merck (Darmstadt, HE, Germany). Triethylamine and trifluoroacetic anhydride (TFAA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified in a Milli-Q system (Millipore, Billerica, MA, USA).

Instrumentation

HPLC analyses were carried out with a Shimadzu® system (Kyoto, Japan) equipped with one LC-20AT pump, a photo diode array detector (SPD-M20A), an autoinjector (Proeminence SIL-20AC) and a column oven (CTO-10AS/VP) controlled by a CBM-20A communication module and LC-Solution software. The elution system was as follows: a Luna C18(2) column (150 × 4.6 mm i.d., 5 µm; Phenomenex, Torrance, CA, USA) with a C18 security guard cartridge (4.0 × 3.0 mm; Phenomenex, Torrance, CA, USA) was eluted in isocratic mode with a mobile phase consisting of 87% phosphoric acid 0.025% + triethylamine buffer pH 3.4 (34:66 v/v); and 13% acetonitrile HPLC grade at a flow rate of 1.0 mL min^{-1} and 20 ºC. Triethylamine buffer (pH 3.4) was prepared by mixing 10 mL of triethylamine with 9 mL of phosphoric acid and water q.s. 1000 mL. The diode array detector (DAD) was set at 210 nm for detection of amphetamine and fenproporex, and 252 nm for detection of amfepramone.

Confirmation of positive cases was performed using a gas chromatograph model GC 2010 coupled with a single quadrupole mass spectra (MS) model QP 2010 (Shimadzu®, Kyoto, Japan).

Urine samples and volunteers

Large truck drivers (over 30 t) were randomly stopped on different highways in the State of São Paulo, Brazil, between April and August 2013 and were asked by police officers to participate in research assessing the prevalence of driving under the influence of drugs or alcohol. As soon as the interview was carried out, the next vehicle was stopped. However, it should be noted that no systematic sampling method was applied for selection of either the survey sites or participants. Between April and August 2013, this sampling method was carried out three times and in each one it was collected around one hundred samples.

This toxicological evaluation was part of an operation called Truck Driver's Health Program, administered by

the Highway Federal Police Department. The program also involved basic dental and clinical evaluations of the drivers and other benefits on interstate roads during the morning. Participation was voluntary and anonymous and all participants signed an informed consent form. The truck drivers also answered a questionnaire consisting of basic information, e.g., age, schooling, and marital status; and use of medicines and recent drug, e.g., cocaine, cannabis, and amphetamines. To ensure the confidentiality of the volunteers, questionnaires and urine samples were collected (plastic bottles) and identified only with a code number. Therefore, it was not possible to match the code number with the identity of the participant.

The protocol of study was previously approved by the Research Ethics Committee of the Clinics Hospital of the Faculty of Medicine of the University of São Paulo (CAPPesq, HC-FMUSP, Ethics Protocol Approval No. 0093/09). Out of 392 truck drivers stopped, 390 (99%) agreed to participate and provided urine samples. Five samples were discarded due to low volume, which was not enough to application of the method. The samples were frozen (−20 ºC) until analysis. Drug-free samples were used for the validation of the method.

Methods

Preparation of standard solutions

Working solutions of AMP, FEN, AMF, and CLP at concentrations of 200, 100 and 20 μg mL^{-1} were prepared with methanol in volumetric glassware. Stock solutions were stored refrigerated (2-8 °C) when not in use.

Sample preparation

An aliquot of 2.0 mL of urine sample was transferred to a plastic tube (10 mL of capacity) containing 1.0 g of NaCl, followed by the addition of 1600 ng of the internal standard CLP (80 μL of a solution of 20 μg mL^{-1}). In each tube, 200 μL of KOH 5 mol L^{-1} and 4 mL of fresh distilled diethyl ether were added. During extraction, the system was submitted to shaking for 10 min, followed by centrifugation at 2000 rpm for 10 min. The supernatant (organic phase) was transferred to a beaker containing 0.5 g of anhydrous sodium sulfate and then to a conical tube with 15 μL of HCl 0.1 mol L^{-1} and dried under nitrogen stream. The residue was reconstituted with 100 μL of the mobile phase and an aliquot of 20 μL was injected into the HPLC-DAD system.

Validation of the method

The method was validated by establishing limits of detection (LOD) and quantification (LOQ), linearity, intra- and inter-assay precision and recovery of the analytes, as described below.[19]

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was defined as the lowest concentration of spiked samples that yields a response greater than the average signal of the negative samples (six sources of blank urine) plus 3.3 times the relative standard deviation (RSD). The LOQ was the lowest concentration that presented a RSD that did not exceed 15% (in six replicates).

Linearity

Linearity is the ability of an analytical method in demonstrating proportional relationship between the method response and concentration of the analyte in the matrix over the range of analyte concentrations of interest (working range). Linearity was examined by analyzing urine samples containing all analytes at the following concentrations: 150, 200, 400, 600, 800 and 1000 ng mL^{-1}. Six replicates were analyzed at each concentration. Homoscedasticity was also checked by a simple one-sided F-test between the variances at the highest and the lowest concentration levels.

Precision and accuracy study

Precision and accuracy were studied by analyzing urine samples spiked with all analytes at concentrations of 250, 500 and 750 ng mL^{-1}. The tests were performed on three different and consecutive days. The analyses were performed in six replicates for each day. Precision, defined as the RSD, was determined by intra- and inter-day repetitions. Experimental concentrations were obtained using the standard calibration curves. Accuracy was expressed as a percentage of the known concentration, i.e., mean measured concentration / nominal concentration × 100.

Recovery

Recovery can be defined as the percentage of the analyte originally present in the specimen that reaches the end of the extraction procedure. The recovery studies were performed by preparing two sets of samples. One set of samples (set A), consisting of three concentrations (250, 500 and 750 ng mL^{-1}) for each drug, was analyzed in six replicates for each concentration, according to the method described in Sample preparation section. The second set (set B), also comprised samples in six replicates for each concentration (250, 500 and 750 ng mL^{-1}). However, for this set, the analytes were spiked into the samples immediately after the LLE procedure. The recovery was calculated by comparing the ratios of analyte peak areas to internal standard (IS) peak areas for the extracted (set A) and unextracted samples (set B).

Application to real samples

Urine samples collected from truck drivers (n = 385), as described in Urine samples and volunteers section, were analyzed according to the developed HPLC method. The quantification was based on the ratios of the peak areas of the compounds to the IS peak areas. The calibration curves were used to determine the amphetamines concentrations. Samples that presented positive results in the HPLC screening method were submitted to another LLE procedure, as described in the Sample preparation section. The residue was derivatized using trifluoroacetic acid anhydride (TFAA). After the reaction and dryness under nitrogen stream, the residue was resuspended with ethyl acetate. An aliquot of 1.0 µL was injected into the GC-MS equipment to confirm the identity of the amphetamines.

Results and Discussion

Despite the development of new techniques, liquid-liquid extraction still predominates in most laboratories when protein-free samples (such as urine) or liquid samples with low protein content (such as serum or plasma) need to be extracted, because this technique is efficient, fast and low cost.[17]

The use of triethylamine as part of the composition of the mobile phase was based on the competition between the solvent and the analytes for the silanol groups of the column, thus avoiding a too long retention time and asymmetric peaks.[20] Amfepramone was analyzed at a different wavelength because of the increase in the observed absorbance with substantial gain in the sensibility.

The LOD and LOQ obtained with this method for all amphetamines were 120 and 150 ng mL^{-1}, respectively; values that are below the cut-off value established by Substance Abuse and Mental Health Services Administration (SAMHSA) for amphetamines screening in workplace drug testing (500 ng mL^{-1}).[21] Other parameters of the validated method (intra- and inter-assay precision, accuracy and recovery) are shown in Table 1.

In the calibration curve range (from 150 to 1000 ng mL^{-1}), the phenomenon of heteroscedasticity was presented (evaluated through the F distribution), probably due to the large range considered in the study of linearity. Therefore, ordinary least square linear regression methods could result in large errors in the calculation of the drugs concentrations, especially in smallest values. By using weighted least squares linear regression, the sum of percentage of relative error (%RE) over the whole range indicated goodness of fit in the evaluation of the effectiveness of the weighting factor used (1 / y) for amphetamine and amfepramone.[22] Other empirical weights, such as 1 / x; 1 / x^2; 1 / $x^{1/2}$; 1 / y^2 and 1 / $y^{1/2}$ were also evaluated. The weighted least squares linear regression equations and coefficients of correlation were: AMP: y = 0.01589x – 0.00018, r^2 = 0.999; FEN: y = 0.0011x + 0.0081, r^2 = 0.998; and AMF: y = 0.001954x + 0.014042, r^2 = 0.999; where y and x represent the relationship between the peak area ratio (compound / IS) and the corresponding calibration concentrations, respectively.

Table 1. Confidence parameters of the validated method for the determination of the amphetamine (AMP), fenproporex (FEN) and amfepramone (AMF) in urine samples

	AMP	FEN	AMF
Intra-day precision (RSD / %)			
C1[a]	2.9	6.2	3.6
C2[b]	2.2	5.0	10.5
C3[c]	2.0	3.4	5.0
Inter-day precision (RSD / %)			
C1[a]	5.2	6.9	4.3
C2[b]	7.8	10.5	11.2
C3[c]	4.5	4.9	5.5
Accuracy / %			
C1[a]	92.0	95.3	86.7
C2[b]	90.7	95.3	89.0
C3[c]	97.7	96.7	97.7
Recovery / %			
C1[a]	57.7	62.3	57.9
C2[b]	57.3	73.3	52.8
C3[c]	82.6	60.7	57.8

[a]250 ng mL^{-1}; [b]500 ng mL^{-1}; [c]750 ng mL^{-1}; RSD: relative standard deviation.

Accuracy data were determined and lay all within the acceptance interval of 15% (20% at the LOQ) of the nominal values for all analytes and concentrations. The recovery values for the studied ATS were all above 50%, value considered suitable for the liquid-liquid extraction procedure.[17]

The developed HPLC-DAD method was applied to 385 urine samples collected from truck drivers who traveled through Brazilian highways. Amphetamine, a fenproporex metabolite, was detected in ten samples of the volunteers and one of them also tested positive for fenproporex. This result can be possibly due to a recent and/or a repeated consumption of the drug.[23,24] Table 2 shows the self-report about amphetamines consumption pattern of the ten participants whose urine samples tested positive for any ATS. Considering the excretion of amphetamines by urine, only one case reported the last use in a period of time that really could be detectable in urine. Two truck drivers also

Table 2. Data of positive cases presenting the self-report of the interviwers (considering amphetamines consumption pattern) and results of toxicological analyses in urine samples

	AMP consumption	Last time	Drug	Age / year	Employment type	Quantification / (ng mL^{-1})
1	Yes, and I still do	1 week ago	not informed	47	autonomous work	AMP: 753
2	Yes, and I still do	1 day ago	manipulated[b]	33	autonomous work	AMP: 809
3	No, I've never	NA[a]	NA[a]	52	autonomous work	AMP: 6184 FEN: 4262
4	Yes, but no longer	3 months ago	Desobesi-M®[c]	28	autonomous work	AMP: 1146
5	No, I've never	NA[a]	NA[a]	28	employed	AMP: 3657
6	Yes, and I still do	2 weeks ago	Desobesi-M®[c]	25	employed	AMP: 2147
7	Yes, and I still do	1 week ago	Dualid S®[d]	32	autonomous work	AMP: 3440
8	Yes, but no longer	4 months ago	manipulated[b]	24	employed	AMP: 557
9	Yes, but no longer	1 year ago	Lipomax®[e] Desobesi-M®[c]	63	employed	AMP: 2555
10	Yes, but no longer	2 months ago	Desobesi-M®[c]	30	employed	AMP: 462

[a]Not applicable; [b]refers to drugs with unknown composition; [c]trade name for drug with fenproporex in their composition, Aché Laboratórios Farmacêuticos S.A.; [d]trade name for drug with amfepramone in its composition, Aché Laboratórios Farmacêuticos S.A.; [e]trade name for drug with fenproporex in their composition, Divcom Pharma; AMP: amphetamine; FEN: fenproporex; n = 10.

said that they had never used any ATS in their whole life, although the toxicological analysis showed a positive result. All this contradiction may be due to the fear of disapproval. The drugs most commonly used and the quantification of the positive cases are also presented in Table 2.

Figure 2 illustrates the result obtained with the application of the developed method to a real sample donated by a truck driver, which was positive for both amphetamine and fenproporex.

As mentioned before, the confirmation of all positive results in HPLC analysis was performed by GC-MS and no false positive results were found. Figure 3 illustrates the full scan mass spectra of derivatized amphetamine and fenproporex. No positive results were found for amfepramone. We believe that the number of amphetamine positive cases was not high due to the appetite suppressant medicines had their production and commercialization forbidden in Brazil in 2011 and the drivers were randomly stopped without any suspicion.

A questionnaire was also applied to the participants by a staff member. All of them were male, and most were married (68.1%) and had only elementary school-level educations (53.5%). The ages of the drivers ranged between 21 and 76 years and the average was 41.1 years old (40.5-41.7, n = 385). At the moment of the interview, the majority (69.6%) of truck drivers were hired by any company. Alcohol consumption was reported by 59.5% of the participants and beer during weekends was the most alcoholic beverage and period mentioned, respectively. Among those who declared to smoke (22.9%), the number of cigarettes consumed *per* day ranged between 1 and 60

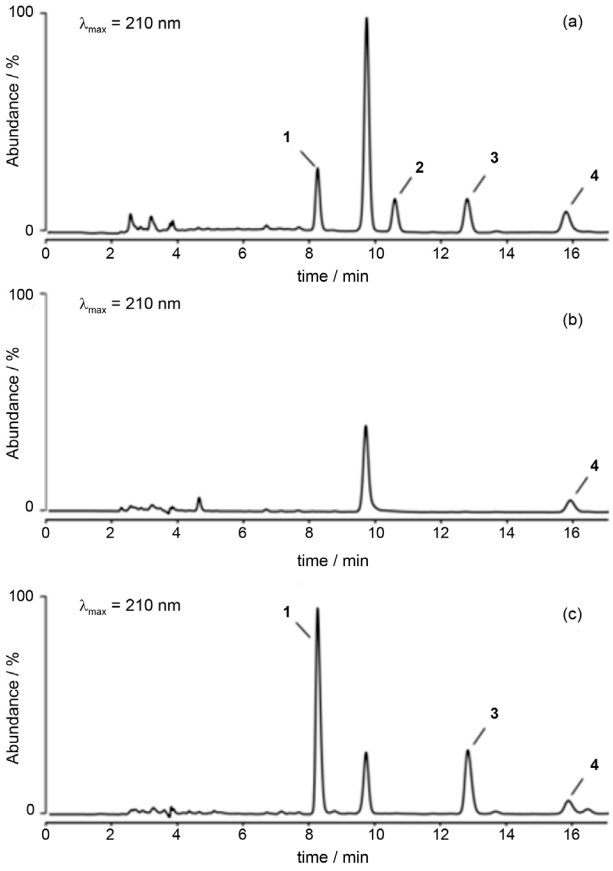

Figure 2. Chromatographic profile obtained by the liquid-liquid extraction (LLE) high performance liquid chromatography with diode array detection (HPLC-DAD) analysis of a urine sample spiked with amphetamine (**1**), amfepramone (**2**), fenproporex (**3**) and internal standard (IS) chlorprenaline (**4**) at a concentration of 1000 ng mL^{-1} (a); drug free urine sample with IS (b); and an urine sample from a truck driver, containing 6184 ng mL^{-1} of amphetamine and 4262 ng mL^{-1} of fenproporex (c).

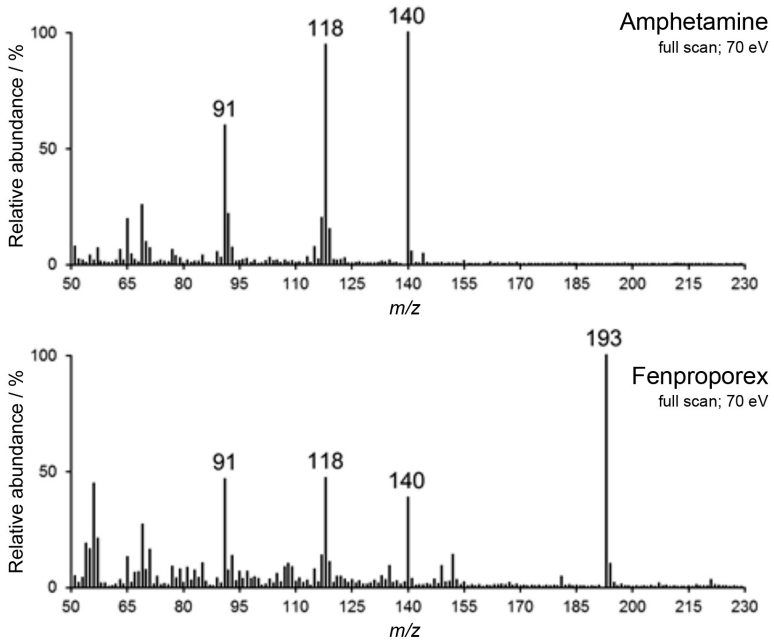

Figure 3. Full scan mass spectra of trifluoroacetyl-derivatives of amphetamine and fenproporex.

and the average was 16. When asked about health problems, 28.8% reported having mainly high blood pressure, diabetes and/or stress. It was also asked if the drivers felt any pain while driving and if they practiced any physical activity: 29.6% referred pain, mainly in hands, shoulders, column, arms, knees, legs and feet; and 44.9% declared to practice physical activity during weekends, like running or soccer.

Conclusions

The developed method proved to be accurate and sensitive, and thus can be used in epidemiological studies and in workplace drug testing. Liquid-liquid extraction and HPLC-DAD detection are well suited to the determination of some amphetamine-type stimulants (amphetamine, fenproporex and amfepramone) in urine samples. The analyses of real samples demonstrated that the consumption of amphetamines really occurs among truck drivers due to different motivation, one of them is their extensive work schedule.

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