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Determination of MDMA, MDEA and MDA in urine by high performance liquid chromatography with fluorescence detection

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Abstract

This paper describes the development and validation of analytical methodology for the determination of the use of MDMA, MDEA and MDA in urine. After a simple liquid extraction, the analyses were carried out on a high performance liquid chromatography (HPLC) in an octadecyl column, with fluorescence detection. The mobile phase using a sodium dodecyl sulfate ion-pairing reagent allows good separation and efficiency. The method showed good linearity and precision. Recovery was between 85 and 102% and detection limits were 10, 15 and 20 ng/ml for MDA, MDMA and MDEA, respectively. No interfering substances were detected with fluorescence detection.
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Keywords: Drug of abuse; MDMA; MDEA

1. Introduction

Each year, we observe a worldwide consumption increase of the well-known synthetic drugs, usually called as designer drugs. The main representatives of this class are "ecstasy" (3,4-methylenedioxy methamphetamine or MDMA) and the "Eve" (3,4-methylenedioxyethyl amphetamine or MDEA), substances with stimulant and hallucinogenous effects. Ecstasy is currently one of the commonly consumed recreational drugs in Europe and USA [1,3].

In Brazil, more than 55,000 tablets were seized during the first half of 2003. According to the Forensic Toxicology Laboratory of the Criminalistic Institute, there was a significant increase in the seizures of that drug in the State of São Paulo, from 2001 to 2002. In 2001, more than 1000 ecstasy tablets were seized. During the first ten months of 2002, this quantity increased to more than 5500 tablets.

There are few articles on this topic in Brazil, and now these drugs have reached a level of importance. It is likely that 20% of individuals who took ecstasy in the mid 90s tried it outside the country, when the drug was not available [2]. Such Brazilian users are usually in the higher socioeconomic classes and can afford such expensive drugs.

Although there is no epidemiological, health studies or overdose reference cases, on this type of abuse, the trends with respect to the illicit ecstasy seizures can be used as indirect indicators of MDMA prevalence and in incidence abuse studies and for other related problems [4].

In the present work, a quick and precise method based on a liquid–liquid extraction and liquid chromatographic separation of urine was developed and validated, allowing the simultaneous detection of MDMA, MDEA through their analysis as well as their biotransformation products, 3,4-methylenedioxyamphetamine (MDA). This method makes possible a simultaneous quantification in a concentration range that makes it possible to distinguish the use of MDMA and MDEA for forensics purposes.

2. Experimental

2.1. Chemicals

MDMA, MDEA, MDA and *N*-methyl-1-(3,4-mehytilenedioxyfenil)-2-butamine (MBDB) solutions (1 mg/ml) were

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purchased from Cerilliant (Round Rock, TX, USA). Working solutions of 100 and 20 μg/ml of each analyte were prepared in methanol with volumetric glassware and used to spike urine samples. Ethyl acetate and *n*-hexane was purchased from Sigma–Aldrich (St. Louis, MO, USA); methanol and acetonitrile were purchased from EM Science (Gibbstown, NJ, USA). All the reagents were of HPLC grade.

2.2. *Instrumentation*

The analyses were carried out on a high performance liquid chromatograph series 1100 HPLC coupled with a model 1046A programmable fluorescence detector (Hewlett-Packard, Little Falls, DE, USA). The excitation and emission wavelengths were set to 285 and 324 nm, respectively. The chromatographic separation was achieved on an Spherisorb ODS2 C18 column (125 mm × 4 mm, 5 μm, Hewlett Packard), maintained at 40 °C during the analysis. The mobile phase, pumped at 1.0 ml/min, was composed of 50 mM solution of sodium dodecyl sulfate in HPLC-grade water, pH adjusted to 4.0 with HCl 1 M (60%) and acetonitrile (40%), in isocratic mode.

2.3. *Sample extraction*

Urine (4 ml) was submitted to a liquid–liquid extraction 5 ml of *n*-hexane/ethylacetate (7/3 (v/v)), after adding 100 μl of methanolic solution of MBDB 20 μg/ml internal standard solution, 2.0 g of NaCl and 0.5 ml of NaOH solution (1 M). Samples were mixed on a shaker for 10 min and centrifuged for 10 min (300 g). The organic phase was transferred to a conical tube containing 50 μl of methanolic HCl (5 M hydrochloric acid in methanol) and evaporated until dry under a fine stream of nitrogen at 30 °C. The residue was reconstituted with 250 μl of mobile phase and a 20 μl aliquot was injected into the HPLC system.

2.4. *Validation of the method*

The method was validated for specificity, linearity, intra- and inter-assay precision, recovery and limits of detection and quantification.

2.4.1. *Specificity*

Six different urine samples were analyzed to determine possible endogenous interferences and were used as “blanks”. The selectivity was studied regarding metabolites, relevant degradation products and some interferences such as caffeine, paracetamol, ketamine, ephedrine, cocaine, amphetamine and methamphetamine.

2.4.2. *Linearity*

Linearity was assessed by analyzing spiked urine samples in six replicates, at the following concentrations: 30, 50, 100, 200, 500, 1000 and 2000 ng/ml.

2.4.3. *Intra- and inter-assay precision*

Precision, defined as relative standard deviation or coefficient of variation (CV), was determined by intra-assays quality controls (QCs) that were prepared by us. They were made by preparing blank urine samples with stock solution, different from that used to prepare the calibrators: 50, 500 and 2000 ng/ml were determined as low, medium and high concentrations, respectively.

2.4.4. *Recovery*

The efficiency of the liquid–liquid extraction was also evaluated through recovery studies performed by preparing two sets of samples of each concentration. One of them (set A) consisting of four samples of 300 and 500 ng/ml concentration and all three analytes were extracted using the aforementioned method. The other one (set B), also consisted of four samples and was extracted exactly the same way as set A, but the standard solution was added to the extract immediately before the residue was reconstituted (unprocessed). For both (set A and B), internal standards were added at the concentration of 500 ng/ml prior to the matrix extraction. The absolute recovery was evaluated by comparison of the average response of the extracted samples spiked before extraction (processed) and the response of extracted blank matrix samples to which analytes had been added at the same concentration immediately before the residue was reconstituted (unprocessed). The unprocessed response represented 100% of recovery [5–7].

2.4.5. *Limit of detection and quantification (LOD and LOQ)*

The limit of detection (LOD) was defined as the lowest concentration at which the results are met at least 90% of the time and still satisfy the predetermined acceptance qualification criteria. The limit of quantification (LOQ) was defined as the lowest concentration that matches all the qualification criteria and ranges ±20% of the target (interpolated value) and presented a CV that did not exceed 20% [7,9].

3. Results

In an attempt to optimize conditions for resolution and efficiency, various mobile phase compositions were evaluated (flow, pH, saline concentration, ion modifiers). It was observed that decreasing the pH and increasing the salt concentration of the buffer of mobile phase was a procedure that is usually sufficient for improving the separation of substances by liquid chromatography but does not result in a satisfactory separation of this amine analytes. The use of gradient elution improved the separation but caused baseline drift and peak broadening of MDA and MDMA. The use of ion-paring salts in the mobile phase was the best solution. Thus, the optimum mobile phase composition used sodium dodecyl sulfate 50 mM (pH 4.0):acetonitrite (60:40).

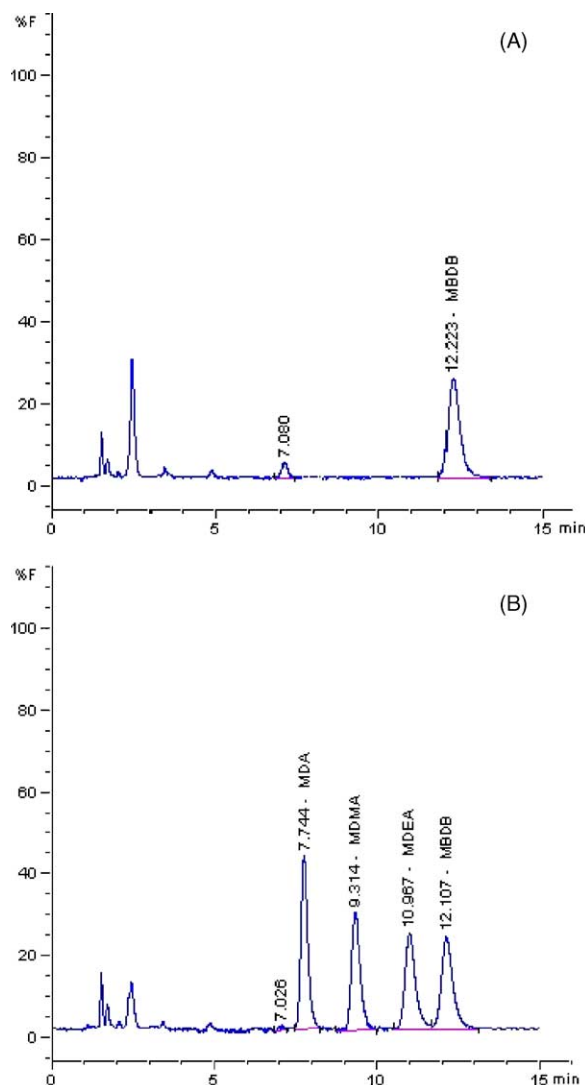

Fig. 1. Chromatogram of blank urine spiked with 500 ng/ml of: (A) MBDB (internal standard) and (B) MDMA, MDEA, MDA and MBDB.

Fig. 1 shows the chromatogram of blank urine spiked with 500 ng/ml of MBDB (internal standard) and a chromatogram of a blank urine sample spiked with 500 ng/ml of MDMA, MDEA, MDA and MBDB. Fig. 2 shows the chromatogram of a positive sample of urine with the presence of MDMA and MDA.

The validation parameters of the method (intra-assay precision, recovery, limit of detection and quantification) for the determination of MDMA, MDEA and MDA are shown in Table 1.

The inter-assay precision was established for the 50 ng/ml QC (n = 6) and were 7.6% for MDMA; 12.2% for MDEA and 8.5% for MDA.

4. Discussion

The increase in the amount of ecstasy seized by the Police in the State of São Paulo and in Brazil are indicative of growth

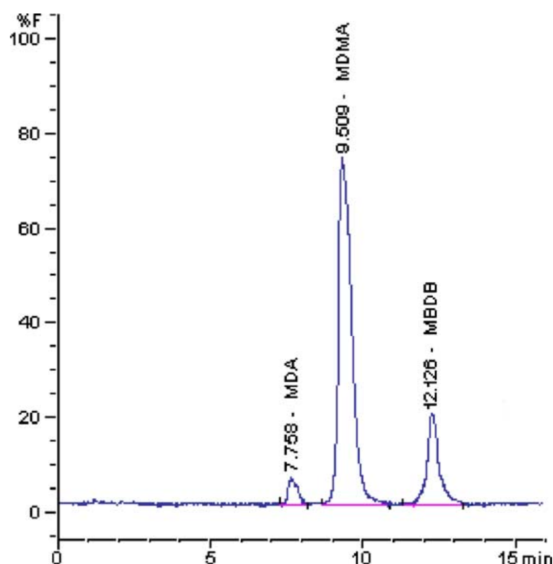

Fig. 2. Chromatogram of urine sample from the Forensic Toxicology Laboratory, MDA (69 ng/ml) and MDMA (1804 ng/ml).

trends in the prevalence and incidence of illicit designer drugs [8]. The corresponding morbidity and even mortality cannot be done unless the appropriate methodology is available.

Urine is the biological sample of choice for establishing use of abusive drugs for forensic purposes, because of, among other reasons, the complexity of matrices like blood and viscera samples, which requires more accurate extraction procedures. The sample preparation techniques, using solid phase micro-extraction (SPME) and headspace sampling, are commonly used to analyze ecstasy. These techniques make use of the semi-volatile methylenedioxylated amphetamines that, after having their heating controlled, are adsorbed in a polydimethylsyloxane fiber (PDMS) [12]. The solid phase micro-extraction, although being simple and clean (practically uses no organic solvents), is not yet routinely used by the forensics laboratories in Brazil, because of economic reasons. The extraction technique proposed here was standardized following the procedures described by other authors [11,14–16], since this method was shown to give the best results, feasibility and was less time consuming. The

Table 1
Confidence parameters of the validated method for the determination of designer drugs MDMA, MDEA and MDA in urine samples by HPLC/FD

	MDMA	MDEA	MDA
Intra-assay precision (CV%)			
50 ng/ml	6.85	12.03	6.69
500 ng/ml	2.05	3.53	2.49
2000 ng/ml	2.84	2.07	3.74
Recovery (%)			
300 ng/ml	102.0 (8.7%)[a]	105.1 (9.4%)[a]	101.2 (5.1%)[a]
500 ng/ml	90.0 (6.3%)[a]	85.5 (3.7%)[a]	98.8 (7.1%)[a]
LOD (ng/ml)	15.0 (25.1%)[a]	20.0 (21.3%)[a]	10.0 (19.9%)[a]
LOQ (ng/ml)	20.0 (16.2%)[a]	30.0 (12.7%)[a]	15.0 (9.0%)[a]

[a] Coefficient of variation (%).

use of the *n*-hexane/ethylacetate (7:3 (v/v)) mixture cited by Clauwaert and coworkers [14–16] gave better results than when the extraction was done only *n*-hexane, as was proposed by Sadegipour and Veuthey (1997) [11]. Also the extraction technique proposed in this study has the advantage of requiring less organic solvent than the procedures cited by the other authors [14–16]. The extraction technique proposed in this paper is simple, fast and has presented good results (the recovery was higher than 85% for all analytes), and is acceptable by forensic laboratory studies.

The relevance of ring-substituted amphetamines in analytical routine of forensic laboratories is well established. The Mandatory Guidelines for Federal Workplace Drug Testing in the United States [21] include the pharmacoidentification of both amphetamine and methamphetamine, but not ring-substituted amphetamines, like MDMA, MDEA and MDA. Consequently, urine immunoassays have not been specifically developed for these compounds and the performance of the existing ones is very poor. Thus, most of the detection procedures involve GC–MS [18]. Although the GC/MS is the technique of choice for the confirmation of these substances, other techniques may be acceptable, providing that the specificity and detection limits for the analytical targets are demonstrated [1,9]. In this context, the high-performance liquid chromatography (HPLC) is a useful technique, mainly because of the lack of a derivatization step and its lower cost, advantages that are very important in developing countries [10].

Initially, we tested the mobile phase proposed by other authors [14–16], but the use of the elution gradient causes baseline drift that decreases the resolution and integration of the peaks. The isocratic mobile phase tested [11] shows low resolution and asymmetrical peaks, probably due to the interaction of the analytes (all secondary amines) with residual silanol, which is present on the silica surface [20]. This led us to seek an alternative mobile phase that further diminishes the interaction among the analytes with the remaining silanol groups of reversed-phase column (since endcapped columns were not available). The use of ion-paring salts makes possible the elution in the isocratic mode and improves the resolution and selectivity of the chromatogram peaks. Furthermore, we did not find any article in which the ion-paring salt was used in the mobile phase.

The HPLC with fluorescence detector technique (HPLC/FD) is among the most used techniques for the analysis of MDMA and analogue compounds. The methylenedioxylated amphetamines, such as MDMA, MDEA, MDA and MBDB are naturally fluorescent, and therefore, detectable by fluorimetric detection, showing high selectivity and sensitivity, which enable quantification at concentrations as low as 10 ng/ml instead of 130–200 ng/ml with the HPLC-UV absorbance detection [10,11]. No endogenous interferences were observed during the analysis of the blank urines. Selectivity of the method was verified, not only with common substances that can appear in seized tablets, such as caffeine, paracetamol, ketamine and ephedrine, but also with some drugs such as cocaine, amphetamine and methamphetamine. None of the several substances tested proved to interfere with the analytes and a very simple chromatogram can be obtained [11,19].

The correlation coefficient (r^2) was higher than 0.98 for the studied dynamic range (50–2000 ng/ml). Samples exceeding the calibration range (in fatal overdose, for example) should be appropriately diluted and re-analyzed [9].

The internal standard used in the analysis was the *N*-methyl-1-(3,4-mehytilenedioxyfenil)-2-butamine (MBDB). That substance has physical–chemical properties very similar to the analyzed substances in this paper, mainly due to the great structural similarity between the internal standard and the analytes. Despite being also classified a designer drug, MBDB is rarely found as an adulterant of ecstasy and Eve tablets, and its presence has never been detected in seized tablets in the State of São Paulo.

The detection and quantification limits were satisfactory considering the analytical system used in the method (extraction and detection system) and were in accordance to the literature data that deal with this matter [11,14]. We found even lower LD and LQ than the reported references [14,15], in which the LQ was 0.1 μg/ml (100 ng/ml).

The values found as LOD, 20, 30 and 15 ng/ml, respectively, for MDMA, MDEA and MDA showed that the method was adequate in the verification of recreational drug use and also because it reaches the necessary values in the confirmation methods recommended by the National Institute of Drug Abuse (NIDA) that declares that the cut-off value for MDMA confirmation in urine is 200 ng/ml [1].

The method is also useful during the drug-use checking for forensics purposes, where sometimes the times for collection exceed those of normal excretion. The urinary excretions of MDMA after the oral administration of 125 mg of the substance were studied, and 24 h after the administration, approximately 50 ng/ml of MDMA were traced according to Ortuño et al. (1999) [13]. The authors also reported that after 24 h, around 50% of the administered MDMA dose was recovered in urine, being a very good biological matrix for verifying ecstasy use. In cases of death caused by ecstasy overdose, the urinary concentration of MDMA may reach 170 μg/ml, and 4 ug/ml of MDA [16]. Weinmann and Bohnert (1998) [17] reported, respectively, 201, 7.1 and 0.135 μg/ml of MDEA, MDA and MDMA urinary concentrations in overdose case.

In addition to LOQ and LOD, this method was successfully evaluated and was linear and precise, having good recovery. Its validation parameters matches the values observed in the literature and allow its application for both the verification of recreational use concentrations and those found in fatal cases of overdose [13,16].

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