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Sensitive and selective determination of methylenedioxylated amphetamines by high-performance liquid chromatography with fluorimetric detection

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Abstract

A rapid, sensitive and selective liquid chromatographic method with fluorimetric detection was developed for the separation and quantification of four methylenedioxylated amphetamines without interference of other drugs of abuse and common substances found in illicit tablets. The method was validated by examining linearity, precision and accuracy as well as detection and quantification limits. Methylenedioxylated amphetamines were quantified in eight tablets from illicit drug seizures and results were quantitatively compared to HPLC–UV analyses. To demonstrate the better sensitivity of the fluorimetric detection, methylenedioxylated amphetamines were analyzed in serum after a liquid–liquid extraction procedure and results were also compared to HPLC–UV analyses. © 1997 Elsevier Science B.V.

Keywords: Amphetamines; Ecstasy; Methylenedioxyamphetamine; Methylenedioxymethamphetamine; Methylenedioxyethamphetamine; N-Methyl-1-(1,3-benzdioxol-5-yl)-2-butanamine

1. Introduction

Methylenedioxylated derivatives of amphetamine such as methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA, Adam, ecstasy), methylenedioxyethamphetamine (MDEA, Eve) or N-methyl-1-(1,3-benzdioxol-5-yl)-2-butanamine (MBDB, Eden) are known as "designer drugs" (Fig. 1) and are considered as illicit substances in many countries [1,2]. Because of the growing abuse of these substances, a number of analytical methods such as immunoassays [3–7], gas chromatography (GC) [3–10], liquid chromatography (LC) [10–18] and more recently capillary electrophoresis (CE) [16,19,20] have been developed for their determination. However, most of these methods allow the determination of only one or two methylenedioxylated amphetamines and rarely include the analysis of MDEA or MBDB. It is therefore desirable to have a complementary method to GC–MS, in order to rapidly determine, without a derivatization procedure, methylenedioxylated amphetamines in seized tablets and in biological fluids.

Recently, we presented a high-performance liquid chromatography (HPLC) method coupled with UV absorbance detection for the determination of six amphetamines (ephedrine, amphetamine, methamphetamine, MDA, MDMA, MDEA) [21]. In the present paper, we show that this method could be applied with few modifications by using fluorimetric

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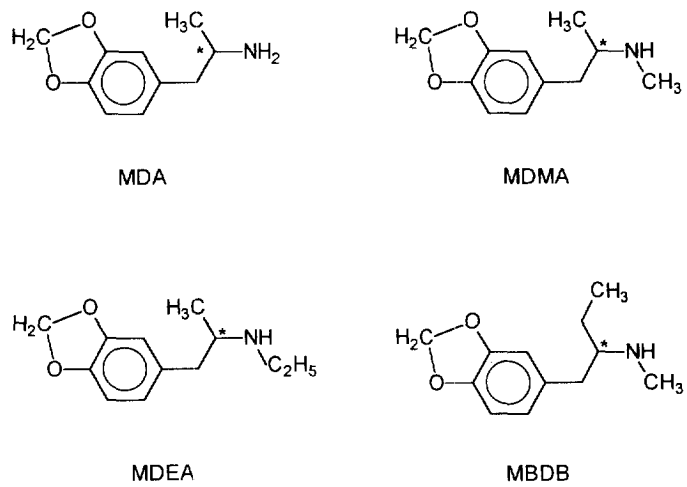

Fig. 1. Chemical structure of the analyzed methylenedioxylated amphetamines.

detection for a more selective, sensitive and rapid determination of only the methylenedioxylated amphetamines. As these compounds are natively fluorescent [13] no derivatization is necessary. Hence, this method can be used for a rapid and selective screening and determination of methylenedioxylated amphetamines. It was validated, compared to our previous HPLC–UV method and applied for the analysis of MDA, MDMA, MDEA and MBDB in seized tablets and spiked serum samples. For these latter, before analyses, we used a classical liquid–liquid extraction (LLE) [22] procedure for amphetamines in biological fluids. To determine the sensitivity of the fluorimetric detection method, analyses were also performed by HPLC–UV method and results were qualitatively compared.

2. Experimental

2.1. Chemicals

Standard solutions of 1 mg ml^{-1} of methylenedioxylated amphetamines (MDA, MDMA, MDEA, MBDB) in methanol were purchased from Alltech (Deerfield, IL, USA). Seized tablets were kindly supplied by the Forensic Institute of Lausanne (Switzerland). Blank human sera from healthy volunteers were obtained from the Clinical Chemistry Laboratory of the Geneva University Hospital (Switzerland). Acetonitrile was purchased from Maechler (Basel, Switzerland). Ultrapure water was supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA). All other reagents, solvents and substances were analytical-grade reagents from Fluka (Buchs, Switzerland).

2.2. Equipment and chromatography

Experiments were carried out on a Gilson HPLC system (Gilson Medical Electronics, Villiers-le-Bel, France), equipped with an ASTED-XL autosampler injector and an oven. The Gilson 712 HPLC software was used for instrument control, data acquisition and data analysis. Detection was carried out using a Merck–Hitachi F-1050 fluorescence detection system (Darmstadt, Germany) operated at an excitation wavelength of 285 nm and an emission wavelength of 320 nm.

The column was a RP18-AB Nucleosil 100, 5 μm (Macherey-Nagel, Oensingen, Switzerland) 125×4 mm I.D thermostatted at 40°C. Mobile phase was composed of a phosphate solution (20 m*M* Na_2HPO_4) adjusted at pH 3.8 with 1.0 *M* HCl and acetonitrile (85:15, v/v). The separation was conducted in isocratic elution mode at a flow-rate of 1 ml min^{-1}. The injection volume was 20 μl.

2.3. Sample preparation

2.3.1. Tablets analyses

Before analysis, each tablet was pulverized into a fine and homogeneous powder. Stock solutions were prepared by dissolving 10 mg of each tablet in 10 ml of 0.1 *M* HCl (this procedure was repeated twice for each tablet). Solutions were sonicated for 30 min to increase solubilities, then vortex-mixed and filtered through 0.45 μm nylon Titan syringe filters (Scientific Resources, Eatontown, NJ, USA). An appropriate dilution of the filtered solutions was injected in duplicate.

2.3.2. Spiked serum samples

Twenty μl of solutions containing 0.5, 2.5 and 10 μg ml^{-1} of MDA, MDMA, MDEA and MBDB were added to 980 μl of blank serum to obtain spiked serum samples at 10, 50 and 200 ng ml^{-1} respectively.

2.4. Liquid–liquid extraction procedure

One ml of spiked serum sample was made alkaline with 200 μl of 1 *M* NaOH solution and the free bases were extracted into 2.0 ml of *n*-hexane by 20 min of rotative mixing. After centrifugation for 6 min at 4000 rpm and freezing the aqueous phase at −4°C, the organic phase was collected and 100 μl of hydrochloric acid–methanol (1:4) were added to convert free amines into hydrochlorides. Then, the solvent was evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of 0.1 *M* HCl and 20 μl were injected into the HPLC system. The extraction was repeated three times and the solution was analyzed by both UV absorbance and fluorimetric detection methods.

2.5. Selectivity

Selectivity of the method was verified not only with common substances which can appear in seized tablets, such as phenylethylamine, caffeine, acetylsalicylic acid, paracetamol, saccharose, lactose, mannitol and sodium chloride, but also with some drugs of abuse such as cocaine, methadone, morphine, phencyclidine, bromazepam, midazolam, diazepam, flunitrazepam, ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, amphetamine, methamphetamine, ethylamphetamine, phentermine, mescaline, 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-methylamphetamine (DOM) and 4-bromo-2,5-dimetoxyphenethylamine (2-CB). All of these compounds were injected in aqueous solution (10 μg ml^{-1}).

2.6. Data analysis

Calibration curves were carried out for MDA, MDMA, MDEA and MBDB at concentrations between 10 and 500 ng ml^{-1} in 0.1 *M* HCl (10.0, 50.0, 100.0, 200.0, 500.0 ng ml^{-1}). Precision and accuracy were tested at 10 and 500 ng ml^{-1} of each methylenedioxylated amphetamine.

Illicit tablets, already analyzed by HPLC–UV [21], were quantified. To compare results obtained by both methods, we applied a Student paired *t*-test evaluated through a standard deviation calculated on paired differences [23,24]. Detection limits (LODs) and quantification limits (LOQs) were expressed as ng ml^{-1} of methylenedioxylated amphetamines injected and based on a signal-to-noise ratio of, respectively, 3:1 and 10:1.

3. Results and discussion

3.1. Fluorescence detection

Previously, we had developed a method with a UV absorbance detection system set at 200 nm [21]. In this study, this method, slightly modified, was used with fluorimetric detection. As mentioned above, only methylenedioxylated amphetamines (MDA, MDMA, MDEA and MBDB) are natively fluorescent and therefore detectable in these conditions. This is why with this more selective method, the acetonitrile proportion can be increased in order to reduce the analytical time. The optimal mobile phase composition was determined as 20 m*M* NaH_2PO_4 solution (adjusted to pH 3.8)–acetonitrile (85:15, v/v). In these conditions, the analysis was performed in less than 6 min (Fig. 2) instead of 14 min by the UV method (t_R = 12.10 min for MBDB which is the last eluting peak).

We observed that the use of fluorimetric detection

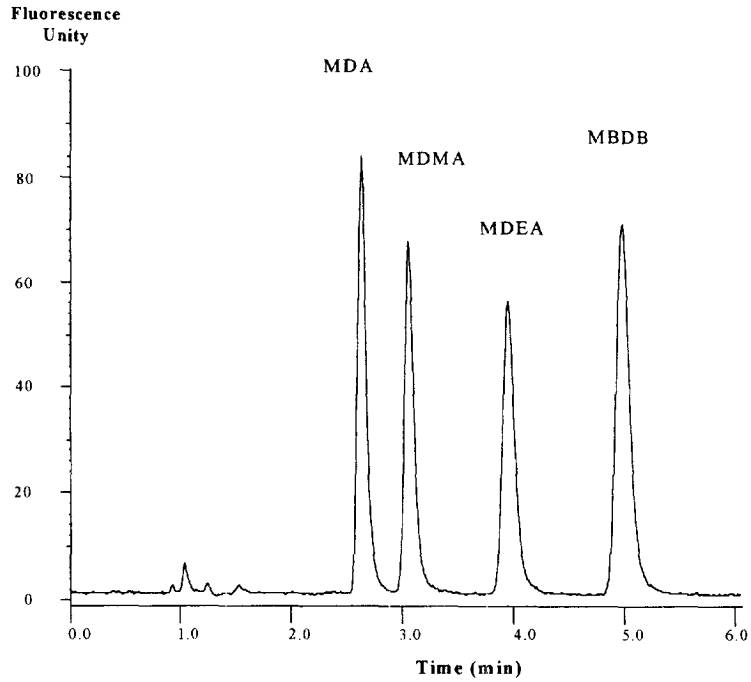

Fig. 2. Separation of four methylenedioxylated amphetamines by HPLC on a Macherey-Nagel column (125×4 mm) C_{18} AB Nucleosil 100 (5 μm); mobile phase: NaH_2PO_4 solution (adjusted to pH 3.8)–acetonitrile (85:15); flow-rate: 1 ml min^{-1}; fluorimetric detection set at Ex: 285 nm, Em: 320 nm.

allowed a very high selectivity. Indeed, none of the several drugs of abuse nor other substances tested for the selectivity interfered with the analytes and, except cocaine (t_R=10.20 min), none gave a chromatographic peak. Indeed, all these substances are not natively fluorescent, which allows to obtain a very simple chromatogram. Furthermore, other recent designer drugs such as DOM, DOB, 2-CB cannot also be detected, because they do not possess the methylenedioxy moiety, avoiding false positive results for methylenedioxylated derivatives.

3.2. Data analysis

We tested the linearity for MDA, MDMA, MDEA and MBDB in the range of 10 to 500 ng ml^{-1}. Correlation coefficients (r) obtained from the plot of experimental values as a function of theoretical values were always greater than 0.999. The intercepts and the slopes were not significantly different from 0.00 and 1.00, respectively (Student t-test, $P<0.05$). Therefore, for all methylenedioxylated amphetamines, the method gave a linear response without systematic errors (fixed or relative). Repeatabilities and reproducibilities were calculated as relative standard deviations (R.S.D.s): repeatabilities were in the range of 0.5–1.8% (500 ng ml^{-1}) and 1.6–10% (10 ng ml^{-1}) and reproducibilities were in the range of 1.0–2.6% (500 ng ml^{-1}) and 12–16% (10 mg ml^{-1}) for six replicate determinations. Furthermore, accuracy of the experimental results was always within 98.9 to 100.9% of the theoretical values (Table 1). These results are in good agreement with those obtained by the previous UV method [21]. However, LOD (2–3 ng ml^{-1}) and LOQ (7–11 ng ml^{-1}) values showed a higher sensitivity for the methylenedioxylated amphetamines.

Indeed, the fluorimetric detection allowed quantitation of methylenedioxylated amphetamines at concentrations as low as 10 ng ml^{-1} instead of 130–200 ng ml^{-1} with the UV absorbance detection. Thus, this method can be applied not only for the direct and rapid determination of these compounds in illicit tablets but also in biological samples.

Table 1
Data analysis of the method for four tested methylenedioxylated amphetamines

Compound	Linear regression equation $y=ax+b$ ($n=10$)		LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Repeatability R.S.D.[a] (%)		Reproducibility R.S.D. (%)		Accuracy (%)
	Slope, b (S.D.)[b]	Intercept, a (S.D.)			500 ng ml^{-1}	10 ng ml^{-1}	500 ng ml^{-1}	10 ng ml^{-1}	
MDA	0.98 (0.02)	0.65 (3.86)	2	7	0.5	1.6	1.5	12	98.9
MDMA	0.99 (0.03)	0.50 (6.19)	3	10	0.5	3.6	1.0	13	100.3
MDEA	0.92 (0.03)	4.12 (6.67)	3	11	1.8	10	2.6	16	100.9
MBDB	0.99 (0.01)	−1.79 (3.22)	3	11	1.1	4.2	2.7	14	98.2

[a] R.S.D.: Relative standard deviation.
[b] S.D.: Standard deviation.

3.3. Application to illicit tablets

Eight tablets (seized by the Swiss Police authorities), quantified previously by HPLC–UV and qualitatively confirmed by GC–MS [21], were analyzed by HPLC with fluorimetric detection. Qualitative results were similar with those previously obtained and Fig. 3 shows the comparison of quantitative results obtained with UV absorbance and fluorimetric detection methods. In this figure, the concentration of the major compound is reported with the mean standard deviation (the homogeneity of the results variances of each method was verified with a Bartlett's test). A statistical test (Student paired differences t-test) was applied to the tablets 1 to 7 which contain the same analyte (MDEA) and

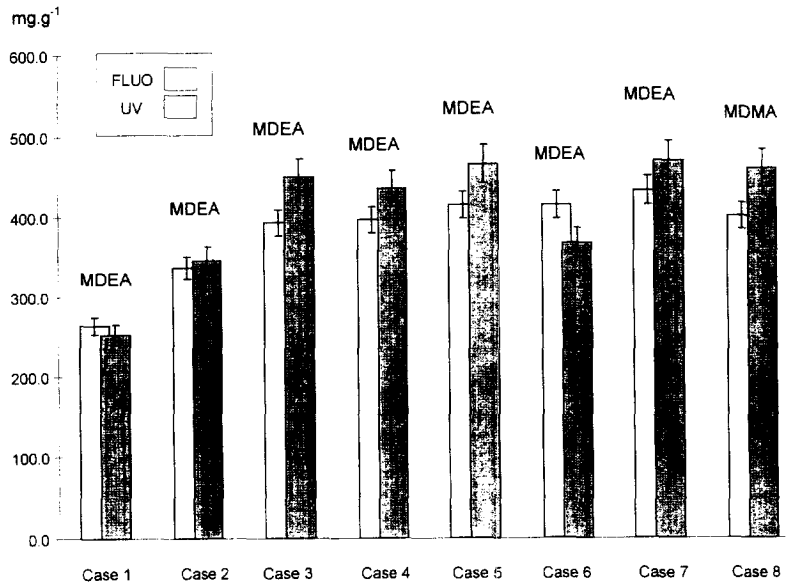

Fig. 3. Comparison of HPLC–UV and HPLC–fluorimetric detection results for eight seized tablets.

confirmed that no significant differences were observed between the two methods. We can note that only one tablet contained ecstasy, while the seven other tablets contained Eve (98–120 mg per tablet) and no MBDB, or MDA were detected.

3.4. Application to spiked serum samples

In order to emphasizes the higher sensitivity of the fluorimetric method in comparison with the HPLC–UV method, we applied both methods to spiked human serum samples. All samples were purified by LLE before analysis. As shown in Table 1 and Fig. 4, the method with fluorimetric detection has a limit of quantification of 10 ng ml^{-1} instead of ca. 200 ng ml^{-1} with the HPLC–UV method for all tested methylenedioxylated amphetamines (taking into account the concentration factor of the LLE procedure). The recovery of the extraction was around 55% (data not shown).

UV absorbance detection could only be applied to fatal cases where postmortem toxicology of lethal cases revealed 110 to 2800 ng ml^{-1} MDMA levels in blood [25]. The highly sensitive fluorimetric detection method is applicable to the selective determination of methylenedioxylated amphetamines in biological fluids even in very low concentrations. A peak plasma MDMA level of 106 ng ml^{-1} was

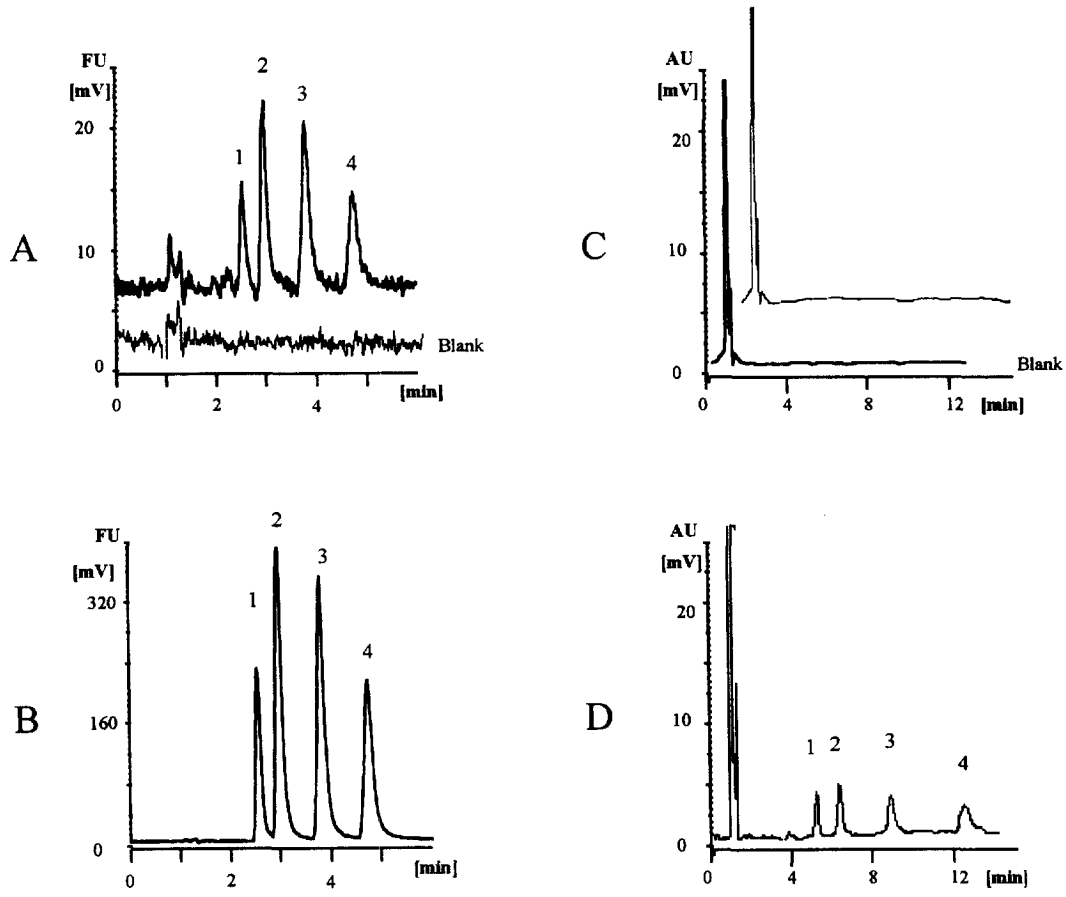

Fig. 4. Qualitative comparison of the sensitvity of HPLC–fluorimetry (A and B) and HPLC–UV (C and D) methods for the analysis of methylenedioxylated amphetamines (1=MDA, 2=MDMA, 3=MDEA, 4=MBDB) in spiked human sera after LLE procedure. (A and C): Blank human serum and human serum spiked at 10 ng ml^{-1} (each amphetamine); (B and D): human serum spiked at 200 ng ml^{-1}.

measured 2 h after oral ingestion of a 50 mg dose; the peak level of the metabolite (MDA) was 28 ng ml^{-1} and occurred at 4 h [26,27].

4. Conclusions

We present a very rapid, sensitive and selective HPLC method coupled with fluorimetric detection for the screening and determination of usual methylenedioxylated amphetamines (MDA, MDMA, MDEA and MBDB). These substances can be analyzed in less than 6 min, with a very high selectivity avoiding any interferences from drugs of abuse or other common substances found in seized tablets or in biological fluids. The method was successfully validated and proved to be linear, precise and accurate. It was applied to the determination of eight seized tablets sold as being "ecstasy". The high sensitivity of fluorimetric detection (i.e., LOQ=10 ng ml^{-1}) also allows the use of this method in the analysis of matrices such as serum where these compounds are present at lower concentrations.

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