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# Simple and reliable high-performance liquid chromatography fluorimetric procedure for the determination of amphetamine-derived designer drugs

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**Abstract**

The paper describes a HPLC–fluorimetric procedure for the determination of methylenedioxyamphetamine, methylenedioxymethamphetamine, methylenedioxyethamphetamine and *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine in urine, serum, saliva and street samples, that features interesting advantages over other procedures previously described. The method requires a very small sample volume (100 μl) and no extraction, lacks matrix effect, and is not time consuming. Linearity was in the range 50–1000 ng/ml regardless of matrix. Sensitivity and detection limit were 50 ng/ml and 10 ng/ml, respectively, but they may reach 10 ng/ml and 2 ng/ml if a slight modification is introduced in the procedure. Intra- and inter-day precision were always within 5% and 8%, respectively. Recovery was satisfactory for all matrices. The described procedure could be successfully used for clinical, epidemiological and forensic applications. 

*Keywords:* Amphetamines; Methylenedioxylated amphetamines

## 1. Introduction

In the last decade, many countries have registered an increasing diffusion of ''designer drugs'', methylenedioxylated derivatives of amphetamine. These drugs, powerful stimulants of the central nervous system, are in great demand, mainly among young people, because they enhance understanding, communicativeness and empathy and produce hallucinogenic effects. Their widespread use has raised concern in Europe and the USA and much has been published [1–3] about their health and social consequences. The most representative substances in this group are MDMA (methylenedioxymethamphetamine or Adam or Ecstasy), MDEA (methylenedioxyethamphetamine or Eve), MDA (methylenedioxyamphetamine) and the MBDB [*N*-methyl-1-(1,3-benzdioxol-5-yl)-2-butanamine, Eden]. These substances are scheduled as illicit drugs in Italian law [4]. For studying and monitoring purposes, many reliable and suitable analytical methods for biological and street samples (e.g., materials from seizures) have been improved [5–11]. Methodological progress is ever more important since many new synthetic drugs can be easily obtained by simple

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chemical substitutions on the same basic structure. As a consequence, identification will often be an awkward problem.

Up to today, screening for amphetamines in biological samples has usually been performed by immunoassay. Gas chromatography–mass spectrometry (GC–MS) is the most widely used method to confirm positive results. Methods like GC with other detection methods, high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), or capillary electrophoresis [12–20] have also been used.

Notwithstanding the great number of publications on this subject, sample preparation and instrumental techniques need to be further improved. Keeping this in mind, we evaluated a HPLC procedure with fluorimetric detection, to assay MDMA and MDA in urine. Our targets were: (i) to verify the reliability of the procedure, (ii) to test its application to amphetamine derivatives other than MDA–MDMA, and (iii) to prove whether the procedure is appropriate for other matrices besides urine.

Some papers described HPLC procedures for MDA, MDMA, MDEA, MBDB with fluorimetric detection [21,22], but the procedure we propose is innovative since it leaves out sample extraction and matrix effect, and allows the simultaneous, rapid detection of different dioxyderivative molecules.

## 2. Experimental

### 2.1. Apparatus

MDA, MDEA, MBDB and MDMA were determined by reversed-phase, isocratic chromatographic analysis with fluorimetric detection. We used HPLC instrumentation by Merck, equipped with an automatic sampler (AS-2000A Merck, Darmstadt, Germany), a fluorimetric detector (Model F1050 Merck–Hitachi) and a computer with the appropriate software to store and process data. Fluorescence detection was operated at 290 nm excitation, and 320 nm emission. The column was a LiChrocart-LiChrospher 100 RP-18 5 μm, 250 mm×4 mm with precolumn LiChrocart-LiChrospher 100 RP-18 5 μm, 4 mm×4 mm (Merck). Flow was 1 ml/min, injection volume 50 μl at room temperature.

### 2.2. Chemicals, standards, samples and controls

The reagents for MDMA–MDA HPLC analysis (MDMA/MDA HPLC method, Cat. No. ECT-LC0050 from Bracco, Milan, Italy) were: reagent 1, concentrated eluent, calibrator 1 (1000 ng/ml of MDA and MDMA) and calibrator 2 (500 ng/ml of MDA and MDMA) in ''stabilized'' urine for the calibration curve. The mobile phase was freshly prepared with 10 ml of concentrated eluent added to 1200 ml of acetonitrile and 300 ml of ultrapure water, degassed under a nitrogen flow before use, and kept at room temperature. The final pH of the mobile phase was 11.4.

Acetonitrile was HPLC grade (Carlo Erba, Milan, Italy). Ultrapure water was obtained by laboratory Milli-Q Unit (Millipore, Bedford, MA, USA).

MDA, MDMA, MDEA hydrochloride 1 mg/ml in methanol were obtained from Sigma (Milan, Italy), MBDB hydrochloride powdered standard was from Lipomed (Arlesheim, Switzerland). These substances were used to obtain standards at 250, 500 and 1000 ng/ml in water, urine, serum and saliva.

Seized tablets, suspected MBDB, were brought in by the police to be analyzed. We tested the procedure on these solid dosage forms.

Drug-free serum, saliva, urine, from healthy, non-using volunteers of the laboratory staff were used to obtain spiked biological samples.

A manufactured human urine multiconstituent control for drug abuse assay (Abbott Labs., Abbott Park, IL, USA), was used to verify selectivity. This control contained benzoylecgonine, morphine, amphetamine, methadone, barbiturates, phencyclidine, benzodiazepines, cannabinoids and was available at low, medium and high concentrations (Table 1).

Table 1
Multiconstituent control content

| Drug | Concentration (ng/ml) | | |
|---|---|---|---|
| | Low | Medium | High |
| Methadone | 300 | 750 | 2000 |
| Amphetamine | 500 | 1500 | 4000 |
| Secobarbital | 300 | 800 | 1500 |
| Benzoylecgonine | 500 | 1500 | 3000 |
| Phencyclidine | 35 | 100 | 250 |
| Nordiazepan | 300 | 600 | 1000 |
| Morphine | 250 | 500 | 800 |
| 11-Nor-δ-9-THC-9-carboxylic acid | 50 | 100 | 150 |

Other substances such as 2,5-dimethoxy-4-methylamphetamine (DOM), 2,5-dimethoxy-4-bromoamphetamine (DOB) and 4-bromo-2,5-dimetoxyphenethylamine (2-CB) were not analyzed because they have been proved not to be detectable by fluorimetric detection [21].

### 2.3. Procedure

Working aqueous standard solutions of MDA, MDEA, MBDB, MDMA were at 1000, 500, 250, 100, 50, 25 and 10 ng/ml.

Mixtures of MDA, MDEA, MBDB and MDMA at different levels were added to each biological matrix and the multiconstituent control to obtain analytical samples at 1000, 500, 250, 100, 50, 25 and 10 ng/ml.

The standard procedure entailed the 1:10 dilution of 100 μl of each analytical sample (i.e., calibrators, standard solutions, spiked biological samples and multiconstituent control) with 900 μl of reagent 1. Then, diluted samples are vortexed, put into capped glass vials and injected (50 μl) by autosampler. Samples at concentrations below 50 ng/ml, i.e.,

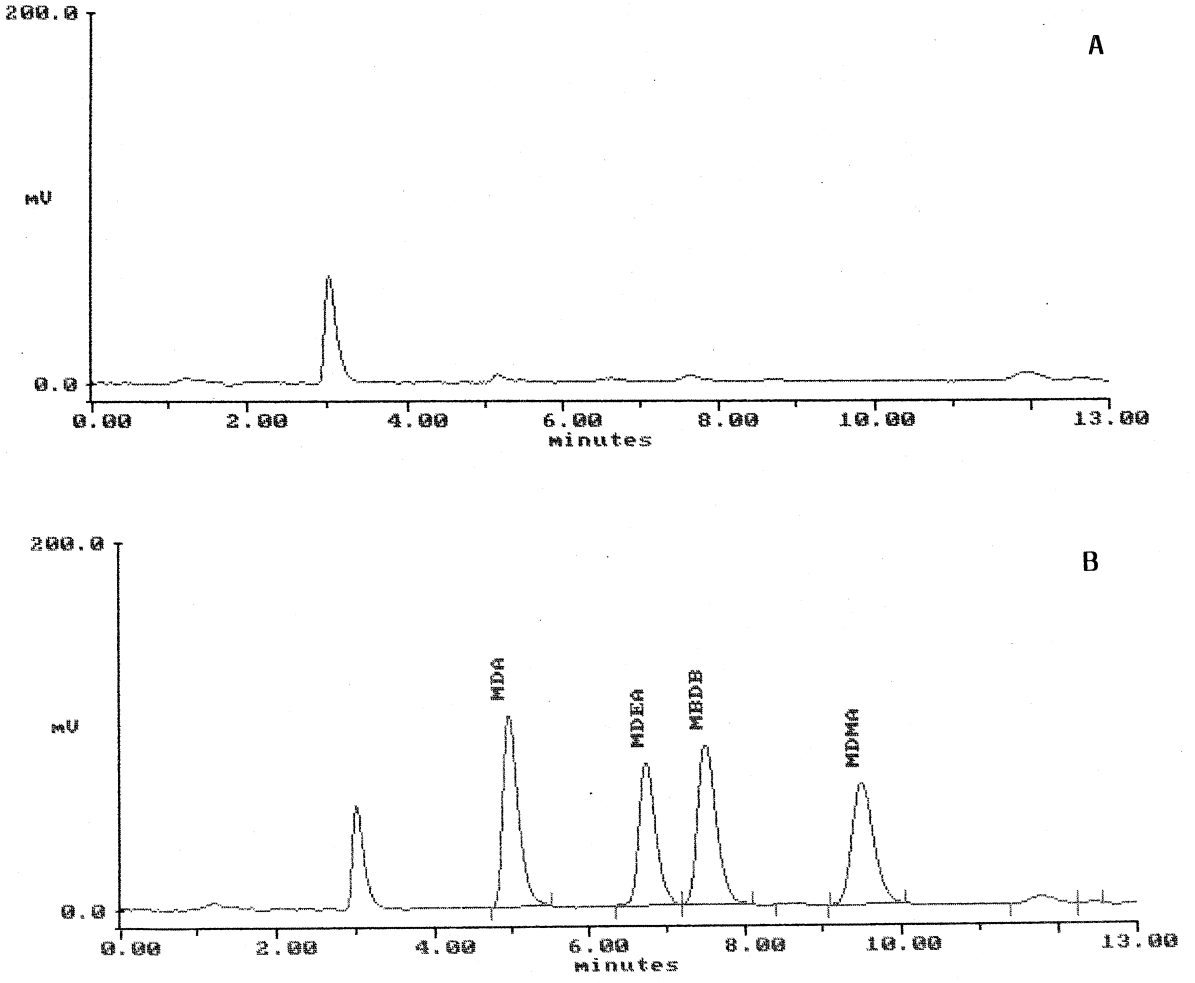


Fig. 1. (a) Chromatograms of (A) blank reagent 1 and (B) standard solutions containing MDA, MDEA, MBDB and MDMA at 1000 ng/ml. (b) Chromatograms of (A) blank urine and (B) urine spiked sample containing MDA, MDEA, MBDB and MDMA at 1000 ng/ml. (c) Chromatograms of (A) blank serum and (B) serum spiked sample containing MDA, MDEA, MBDB and MDMA at 1000 ng/ml. (d) Chromatograms of (A) blank saliva and (B) saliva spiked sample containing MDA, MDEA, MBDB and MDMA at 1000 ng/ml.

below the sensitivity threshold of the method, were directly assayed without dilution with reagent 1 (modified procedure). The use of a precolumn limited the impact of directly injecting biofluids on column life and performance. When an undiluted sample was processed, the precolumn was changed more frequently, i.e., after 30 injections instead of 50 as usual.

Each MBDB tablet was finely pulverised and added to 1 ml of methanol. An aliquot from this suspension was sufficiently diluted ($10^{-6}$ with ultrapure water) to fall into linearity range and then processed as the other samples.

Quantitation of MDA, MDEA, MBDB and MDMA levels in the samples was calculated by comparing their peak areas to calibration curves on water, urine, serum, saliva standards at 250, 500 and 1000 ng/ml.

## 3. Results

The analysis of the mixed substances demonstrated that, under the defined conditions, peaks of MDA, MDEA, MBDB and MDMA appear sharp and well resolved. Fig. 1(a) shows typical chromatograms from blank reagent and a standard solution containing the four substances at 1000 ng/ml. Fig. 1(b), (c), and (d) show blank and spiked biofluids (urine,

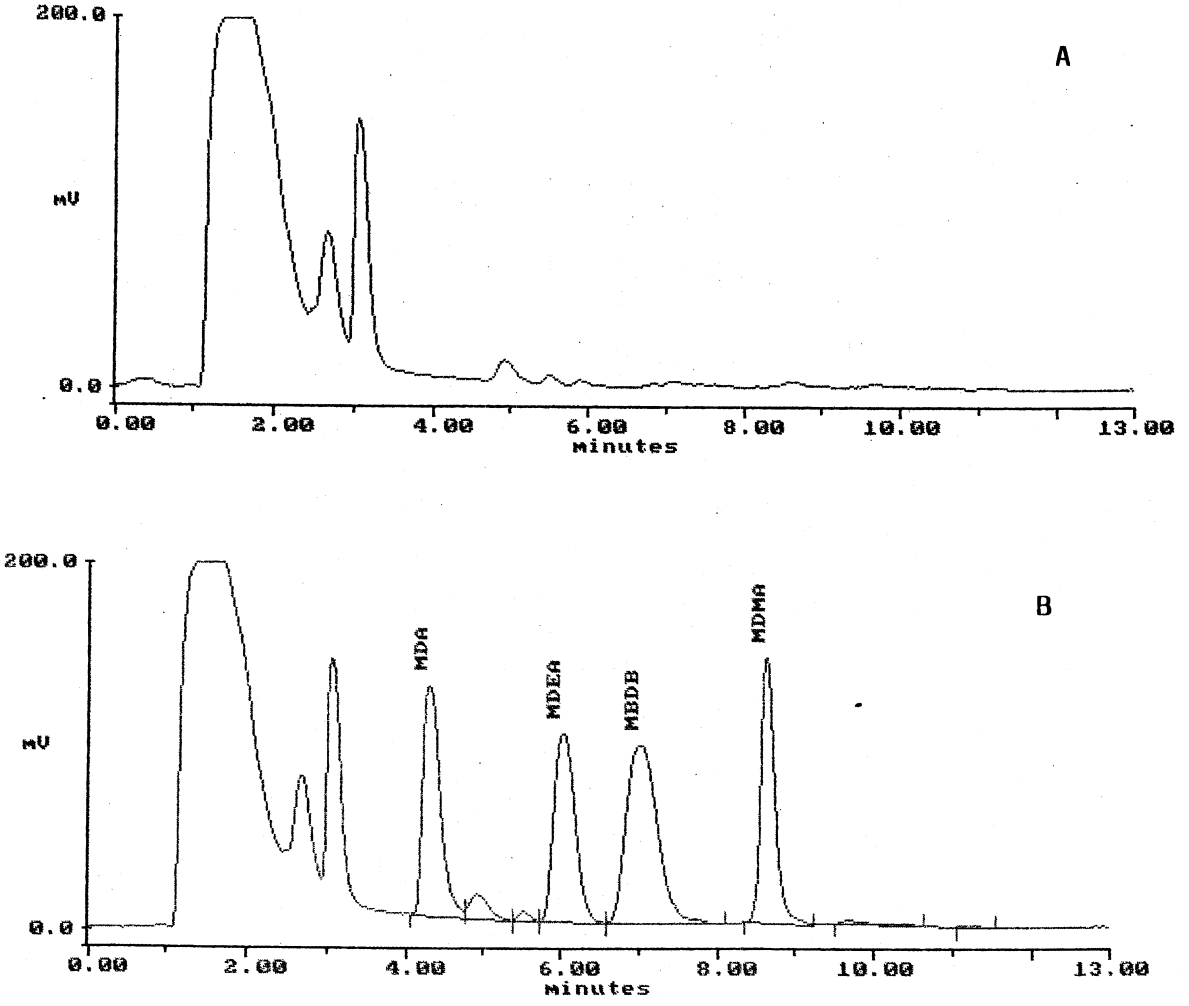


Fig. 1 (b) (*continued*).

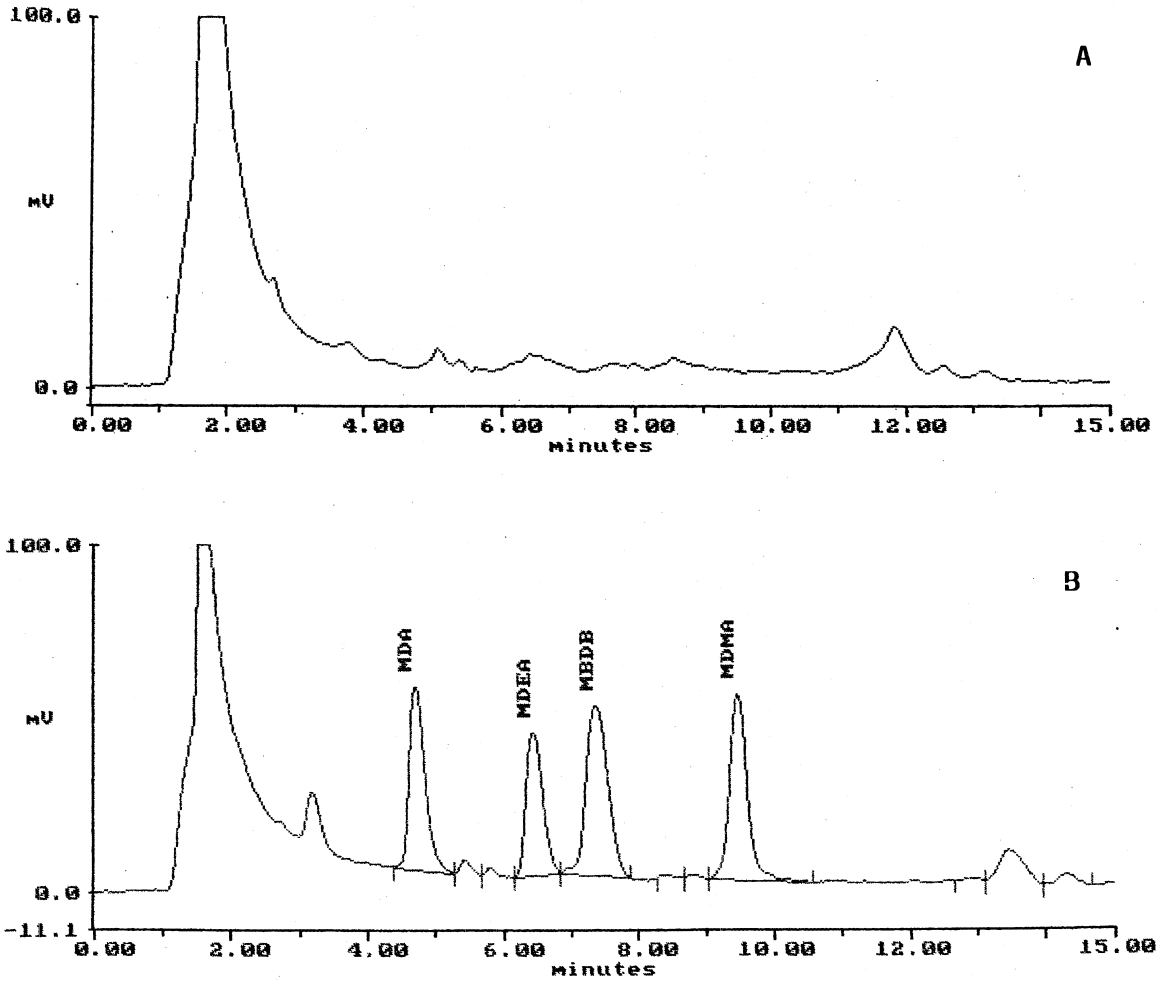


Fig. 1 (c) (*continued*).

serum, saliva) containing 1000 ng/ml of MDA, MDEA, MBDB and MDMA. Table 2 shows mean retention times and their standard deviation (SD) in different samples assayed according to the standard procedure, i.e., diluted with reagent 1. The retention time variation for each sample was not dependent on concentration, and standard deviation never exceeded 0.02 min.

The linearity of the procedure was proved up to 1000 ng/ml in every biological matrix and up to 10 000 ng/ml only in the standard solution.

Table 3 reports the parameters of the linear

Table 2
MDA, MDEA, MBDB, MDMA retention times (min) and standard deviations (SDs) in different matrices

| Standard | MDA | | MDEA | | MBDB | | MDMA | |
|---|---|---|---|---|---|---|---|---|
| | $t_R$ | SD | $t_R$ | SD | $t_R$ | SD | $t_R$ | SD |
| Solution | 4.94 | 0.01 | 6.77 | 0.01 | 7.53 | 0.02 | 9.50 | 0.02 |
| Urine | 4.35 | 0.01 | 6.03 | 0.01 | 6.96 | 0.01 | 8.84 | 0.02 |
| Serum | 4.65 | 0.02 | 6.39 | 0.02 | 7.29 | 0.01 | 9.39 | 0.02 |
| Saliva | 4.53 | 0.01 | 6.23 | 0.01 | 7.12 | 0.02 | 8.85 | 0.01 |

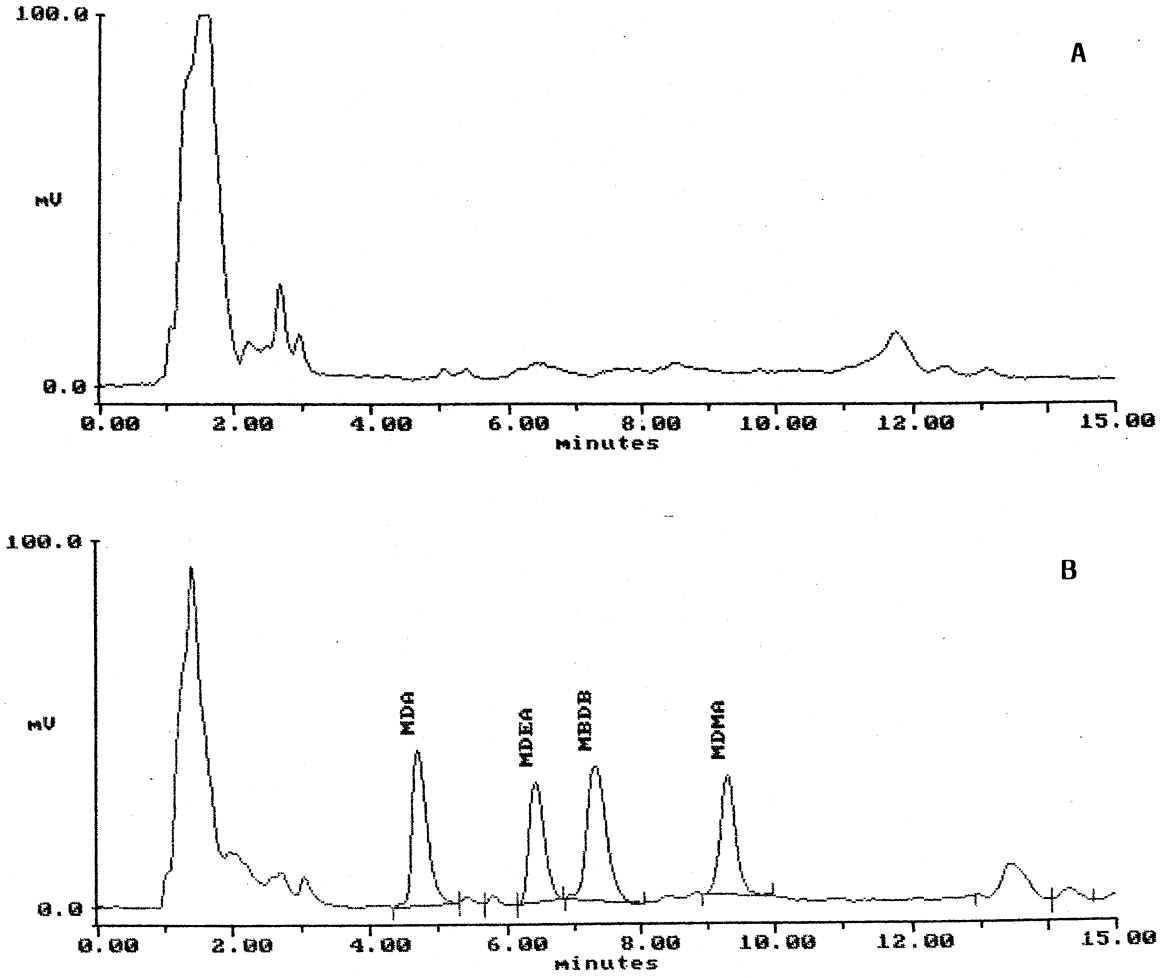


Fig. 1 (d) (*continued*).

Table 3
Linearity of HPLC procedure for the substances in different matrices standard procedure. (range 50–1000 ng/ml)

| | MDA | | | | MDEA | | | | MBDB | | | | MDMA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Slope, *b* (S.E.)[a] | Intercept, *a* (ng/ml) | *Sy·x*[b] | $r^2$ | Slope, *b* (S.E.) | Intercept, *a* (ng/ml) | *Sy·x* | $r^2$ | Slope, *b* (S.E.) | Intercept, *a* (ng/ml) | *Sy·x* | $r^2$ | Slope, *b* (S.E.) | Intercept, *a* (ng/ml) | *Sy·x* | $r^2$ |
| Aqueous solutions | 1.00 (0.024) | 0.22 | 19.03 | 0.98 | 1.00 (0.056) | 0.16 | 44.12 | 0.98 | 1.00 (0.069) | 0.01 | 53.82 | 0.98 | 1.00 (0.073) | −1.00 | 56.73 | 0.96 |
| Urine | 0.99 (0.067) | 0.00 | 51.62 | 0.96 | 1.00 (0.068) | 0.04 | 60.03 | 0.98 | 0.98 (0.099) | −0.04 | 76.23 | 0.94 | 1.02 (0.083) | −0.25 | 63.64 | 0.96 |
| Serum | 0.93 (0.107) | 36.45 | 81.88 | 0.92 | 0.97 (0.099) | 23.79 | 75.49 | 0.94 | 0.98 (0.123) | 19.95 | 94.01 | 0.90 | 0.98 (0.037) | 15.08 | 28.71 | 0.98 |
| Saliva | 1.01 (0.058) | −3.79 | 44.86 | 0.98 | 1.01 (0.070) | 9.50 | 53.50 | 0.96 | 0.99 (0.076) | 4.58 | 58.42 | 0.96 | 1.02 (0.112) | −13.83 | 85.16 | 0.92 |

[a] S.E.=Standard error of the slope.
[b] *Sy·x*=Standard deviation about the regression line.

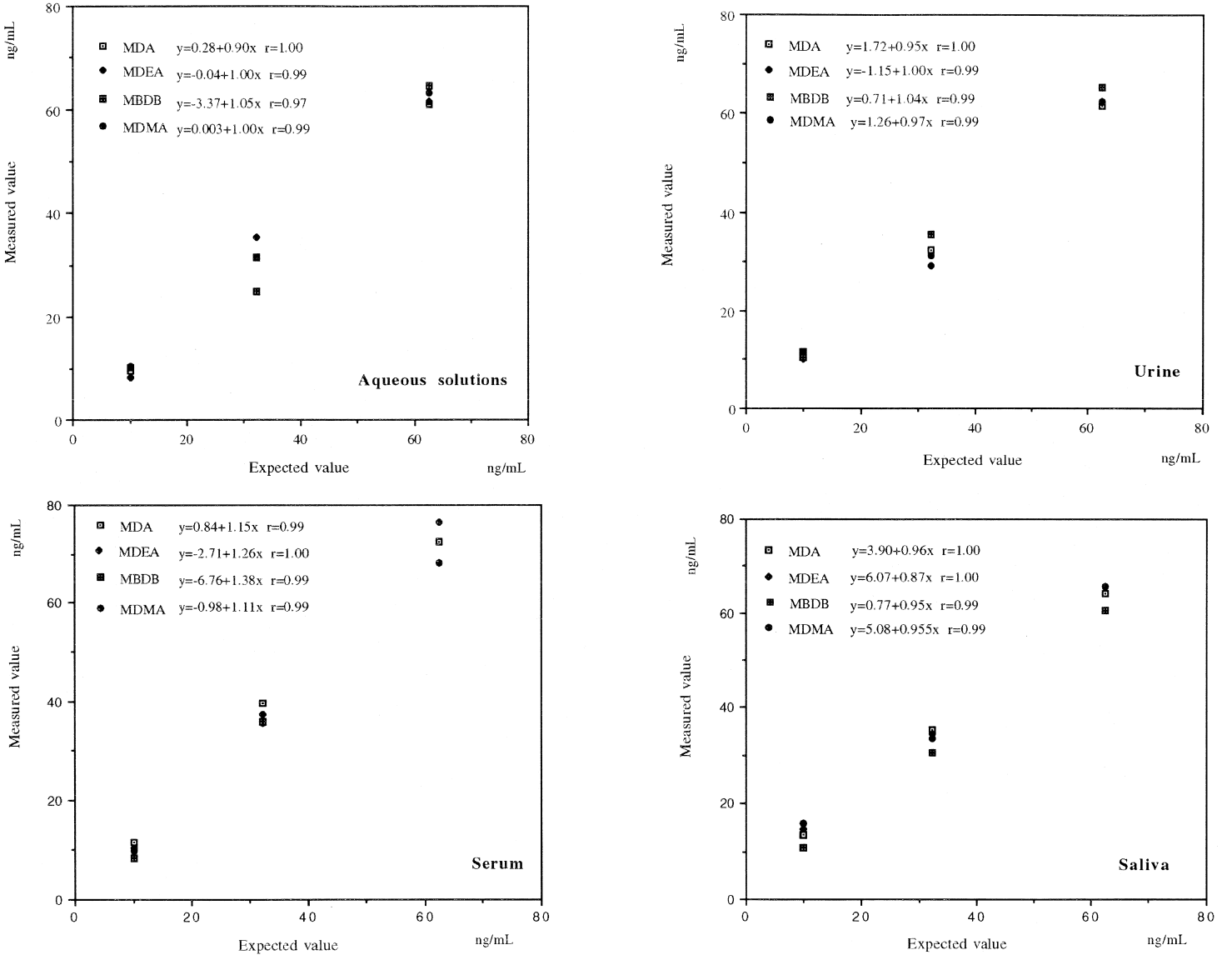


Fig. 2. Analysis of undiluted standard solutions and biofluids (urine, serum, saliva), range 10–60 ng/ml: plotted results.

Table 4
Precision and accuracy of the HPLC standard procedure

| | Expected value (ng/ml) | Aqueous solutions | | | |
|---|---|---|---|---|---|
| | | Mean value (ng/ml) ($n$=15) | RSD (%) | | Mean recovery (%) |
| | | | Intra-day ($n$=5) | Inter-day ($n$=15) | |
| MDA | 250 | 261.2 | 3.5 | 6.3 | 104.5 |
| | 500 | 485.0 | 2.7 | 3.9 | 97.0 |
| | 1000 | 988.7 | 2.6 | 3.7 | 98.9 |
| MDEA | 250 | 249.2 | 1.1 | 5.3 | 99.7 |
| | 500 | 494.0 | 1.8 | 5.0 | 98.8 |
| | 1000 | 997.5 | 2.1 | 2.9 | 99.8 |
| MBDB | 250 | 281.0 | 4.9 | 5.1 | 112.4 |
| | 500 | 506.3 | 2.1 | 5.6 | 101.3 |
| | 1000 | 998.8 | 2.4 | 3.5 | 99.9 |
| MDMA | 250 | 252.7 | 2.9 | 4.6 | 101.1 |
| | 500 | 490.5 | 3.6 | 4.7 | 98.1 |
| | 1000 | 999.8 | 2.4 | 4.6 | 99.9 |

regression analysis in aqueous solution and biological fluids for each substance assayed (five concentration points, 50, 100, 250, 500 and 1000 ng/ml). The procedure was linear in all the matrices. Serum showed a slope standard error nearly always greater than the other matrices, clearly seen in the large intercept. This is attributable to the poor performance of the method at the lowest values when calculated within a wide concentration range.

Sensitivity, determined by six progressive dilutions, was 50 ng/ml. The detection limit of the procedure, i.e., the lowest concentration resulting in

Table 5
Precision and accuracy of the HPLC standard procedure

| | Expected value (ng/ml) | Urine spiked samples | | | | Serum spiked samples | | | | Saliva spiked samples | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean value (ng/ml) ($n$=15) | RSD (%) | | Mean recovery (%) | Mean value (ng/ml) ($n$=15) | RSD (%) | | Mean recovery (%) | Mean value (ng/ml) ($n$=15) | RSD (%) | | Mean recovery (%) |
| | | | Intra-day ($n$=5) | Inter-day ($n$=15) | | | Intra-day ($n$=5) | Inter-day ($n$=15) | | | Intra-day ($n$=5) | Inter-day ($n$=15) | |
| MDA | 250 | 225.8 | 2.3 | 5.1 | 90.3 | 263.6 | 2.6 | 6.3 | 105.4 | 255.3 | 1.9 | 3.3 | 102.1 |
| | 500 | 548.4 | 2.4 | 3.0 | 109.7 | 524.5 | 1.9 | 3.8 | 104.9 | 449.1 | 1.6 | 6.0 | 89.8 |
| | 1000 | 987.0 | 1.2 | 2.5 | 98.7 | 943.6 | 1.9 | 2.4 | 94.4 | 1030.5 | 2.0 | 3.1 | 103.0 |
| MDEA | 250 | 236.6 | 2.1 | 7.5 | 94.4 | 229.9 | 1.3 | 3.5 | 91.9 | 274.6 | 1.0 | 5.1 | 109.8 |
| | 500 | 535.6 | 1.8 | 2.7 | 107.0 | 487.8 | 1.2 | 4.7 | 97.6 | 490.2 | 1.5 | 4.2 | 98.0 |
| | 1000 | 998.2 | 1.6 | 3.2 | 99.8 | 947.6 | 2.6 | 2.9 | 94.8 | 1038.1 | 2.2 | 3.3 | 103.8 |
| MBDB | 250 | 220.2 | 2.3 | 4.5 | 88.1 | 263.9 | 2.2 | 4.0 | 105.6 | 265.5 | 3.5 | 4.8 | 106.2 |
| | 500 | 510.6 | 2.8 | 2.8 | 102.1 | 587.2 | 2.3 | 6.9 | 117.4 | 451.8 | 3.2 | 5.7 | 90.4 |
| | 1000 | 1008.4 | 1.8 | 4.3 | 100.8 | 941.3 | 3.4 | 6.3 | 94.1 | 1043.8 | 2.2 | 3.4 | 104.4 |
| MDMA | 250 | 227.2 | 3.0 | 6.5 | 90.8 | 247.7 | 4.3 | 7.2 | 99.1 | 234.1 | 3.7 | 6.1 | 93.6 |
| | 500 | 526.4 | 1.4 | 3.5 | 105.3 | 491.8 | 2.1 | 3.7 | 98.4 | 445.2 | 2.6 | 4.9 | 89.0 |
| | 1000 | 996.2 | 1.8 | 3.1 | 99.6 | 984.6 | 1.2 | 2.8 | 98.5 | 1029.6 | 1.5 | 3.3 | 102.3 |

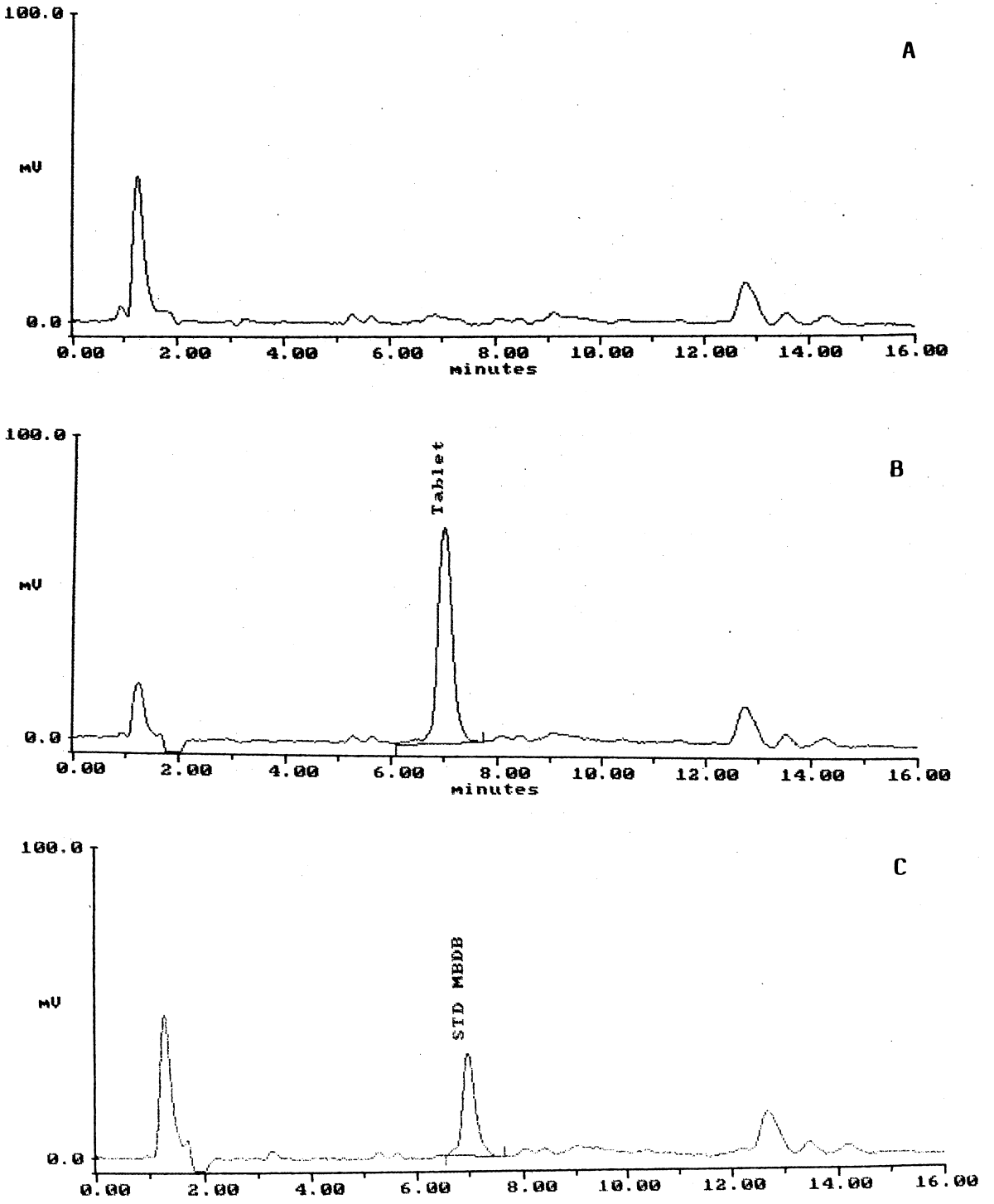


Fig. 3. Chromatograms of (A) blank reagent 1, (B) seized tablet and (C) MBDB standard.

a reproducible signal well separated from the noise, was 10 ng/ml. When the samples were processed undiluted (Fig. 2), sensitivity reached 10 ng/ml and the detection limit was 2 ng/ml.

Intra- and inter-day precision were evaluated measuring for three consecutive days five replicates of each sample at low, medium and high concentrations (250, 500 and 1000 ng/ml) of MDA, MDEA, MBDB, MDMA. Tables 4 and 5 show the relative standard deviations (RSDs) in standard solution, urine, serum and saliva: they were always within 5% and 8% for intra- and inter-day, respectively. The RSDs of undiluted samples for concentrations 100 ng/ml or lower were not reported since they were not different from the corresponding diluted samples. The accuracy in terms of recovery was generally satisfactory despite the differences between standard solutions and biofluids (Tables 4 and 5).

The selectivity of the procedure was checked on the three levels of spiked multiconstituent controls containing benzoylecgonine, morphine, amphetamine, methadone, barbiturates, phencyclidine, benzodiazepines, cannabinoids. None of the substances interfered with the procedure.

No interference was seen in the analysis of seized tablets either. The presence of MBDB in this matrix was confirmed and quantified by comparison with the MBDB standard. Fig. 3 shows chromatograms of (A) the blank reagent 1, (B) a seized tablet, and (C) MBDB standard.

## 4. Discussion

Although a great number of countries are currently doing research into synthetic drugs, much work remains to be done in the analytical field. The development of new GC and LC procedures was stimulated by the false positive results obtained with immunoassay screenings. GC–MS provides the highest level of confidence in the result besides being the most widely used technique for the structural identification of these compounds but its high cost and the high level of operator experience required restrict its use to confirmation analyses.

Because of its widespread use and practicability, we developed a HPLC procedure suitable for different matrices and methylenedioxylated amphetamines, which has several advantages. Sample extraction is not required. This reduces analysis time, sources of errors and operator contact with potentially infectious samples. In addition, substances are not lost as typically happens with any extraction procedure requiring evaporation to dryness, especially for volatile substances such as MDMA. To solve this problem some authors have suggested addition of a small amount of hydrochloric acid [7] to the analytical sample, which involves a more elaborate procedure. In our case, this is not necessary.

The procedure is suitable for different biological fluids. It was linear in all the observed matrices in the range 50–1000 ng/ml for MDA, MDEA, MBDB and MDMA. When concentrations under 250 ng/ml are explored, the use of a calibration curve between 50 and 250 ng/ml is recommended to improve the performance of the method at the lowest concentration values. For concentrations lower than 50 ng/ml, we performed the assay according to the modified procedure, i.e., using the sample without dilution.

The stated characteristics could encourage a more widespread determination of amphetamine derivatives to improve clinical and epidemiological studies on different matrices.

Urine is currently the most common diagnostic medium for drug abuse screening purposes; as a consequence the analytical methodology is not always suitable for other biological fluids. The procedure we propose solves this problem, as shown by the results, and can be used regardless of matrix. In particular, there is an increasing interest in the use of saliva as a diagnostic medium for drugs of abuse, because it can be obtained non invasively, consistent with protection of privacy, without possibility of adulteration. These characteristics are particularly important for road safety applications. Studies [23] regarding amphetamine and methamphetamine have found that the high amphetamine levels found in saliva and the strong effect of urinary pH on drug excretion suggest saliva as the medium of choice for diagnostic evaluation. Although several papers [24–28] report the possible use of saliva and its suitability in drug abuse diagnosis, more systematic studies are needed and, above all, the relationships between saliva and biological fluids, and between dose and time elapsed after intake have to be better understood.

The sensitivity (10 ng/ml) of the proposed HPLC procedure allows the detection of positives in biological fluids several hours after intake, thus enhancing the ability to detect occasional users. Few studies describe the metabolism of these substances in human subjects. Sadeghipour and Veuthey [21] reported that an oral dose of 50 mg of MDMA gave a peak of 106 ng/ml of MDMA after 2 h and 28 ng/ml of MDA after 4 h in plasma [29]; Brunnenburg et al. [22] reported that oral doses of 100–140 mg of MDE·HCl resulted in maximum plasma concentrations of 235–465 ng/ml of MDE and 7–33 ng/ml of MDA after 2 h and, 54 ng/ml of MDE and 16 ng/ml of MDA after 24 h.

None the tested drugs of abuse interfere with fluorimetric detection. Other designer drugs as DOM, DOB and 2-CB cannot be detected [21] since they do not possess the methylenedioxy moiety.

As far as we know, two very recent papers have suggested HPLC fluorimetric procedures to detect methylenedioxy derivatives of methamphetamine with sensitivities around 10 ng/ml in serum and plasma [21,22]. In comparison, the procedure we propose, besides showing a comparable sensitivity and selectivity for dioxylated amphetamines natively fluorescent, presents further advantages. The analysis can be performed on a very small sample volume (100 μl, i.e., one tenth of the volume usually processed), on different matrices including seized material from the illicit market and without sample extraction. The procedure is reliable, simple and can be completed in less than 15 min.

In consideration of the above, this procedure can be easily and advantageously applied to epidemiological and clinical studies.

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