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# Liquid chromatography–atmospheric pressure ionization electrospray mass spectrometry determination of "hallucinogenic designer drugs" in urine of consumers

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**Abstract**

A procedure based on liquid chromatography–mass spectrometry (LC–MS) is described for determination of 3,4-methylenedioxymethamphetamine (MDMA), 2,5-dimethoxy-4-methyl-phenethylamine (2C-D), 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B), 1-(8-bromo-2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b'] difuran-4-yl)-2-aminoethane (2C-B-Fly), 4-ethylthio-2,5-dimethoxy-β-phenethylamine (2C-T-2), 4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I), and 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E), 1-(m-chlorophenyl)piperazine (m-CPP), 4-hydroxy-*N*,*N*-diisopropyltryptamine (4-OH-DIPT) and 4-acetoxy-*N*,*N*-diisopropyltryptamine (4-acetoxy-DIPT) in urine of consumers using 3,4 methylendioxypropylamphetamine (MDPA) as internal standard.

Sample preparation involved a solid-phase extraction procedure at pH 6 of both non-hydrolyzed and enzymatically hydrolyzed urine samples. Chromatography was performed on a $C_{18}$ reversed-phase column using a linear gradient of 10 mM ammonium bicarbonate, pH 7.3 and acetonitrile as a mobile phase. Separated analytes were determined in LC–MS single ion monitoring mode using an atmospheric pressure ionization–electrospray ionization (ESI) interface. The assay was tested on urine samples from consumers of compounds under investigation ($n = 32$).

Limits of quantification varied between 20 and 60 ng/mL for the different analytes under investigation. Calibration curves were linear to 2000 ng/mL for all the substances under investigation, with a minimum $r^2 > 0.99$. At three concentrations spanning the linear dynamic range of the assay, mean recoveries ranged between 55.4 and 95.6% for the different analytes. Higher analytes concentrations in hydrolyzed samples showed the presence of conjugated compounds in urine.


*Keywords:* Hallucinogen; Designer drugs; LC–MS; Urine

## 1. Introduction

Designer drug is a term that includes a number of psychoactive substances which are created to get around existing drug laws by modifying their molecular structures to varying degrees. The term gained popularity in the 1980s when 3,4-methylenedioxymethamphetamine (MDMA) was firstly introduced in the black market [1].

Since late 1990s and early 2000s, new synthetic substances have been introduced in the illicit drug market being sold over internet web sites [2]. Most suppliers purchased these new designer drugs in bulk form as powder, not as pills to support the claim that they were being sold for non-consumptive research and to avoid the legal treatment of controlled substances analogues present in the "U.S. Controlled Substances Act" and in similar banning laws in Europe [3].

These new designer drugs generally present structural features of phenethylamine, piperazine and tryptamine derivatives

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and are reported to produce hallucinogenic/visual effects similar to those of LSD and mescaline and emotional/empathic responses similar to those of MDMA [4–6].

Special concerns relate to the lack of scientific knowledge about pharmaco-toxicology of these compounds in humans and the specific harmful effects of the substances when taken alone or in combination with other drugs. There are a number of reports concerning acute intoxications associated to the consumption of the piperazine derivative meta-chlorophenylpiperazine (m-CPP), the triptamine derivative Foxy or 5-MeO-DiPT and a fatality involving the phenethylamine 2C-T-7 that support the view that consumption of these "hallucinogenic designer drugs" is likely to be a threat to human health [7–12].

When facing cases of supposed intoxications with "hallucinogenic designer drugs", clinical laboratories are confronted with biological samples that may contain a great variety of psychoactive substances. Some of them come into fashion for a short period of time and become outdated very quickly. Therefore, there is a need of developing analytical methodology able to identify a number of varying substances in the most straightforward way. The relatively low amount of samples to be screened for as well as their chemical heterogeneity prevented the development of immunological assays as cost-effective analytical approach. In clinical and forensic toxicology, methodologies involving liquid chromatography (LC) coupled to mass spectrometry (MS) as detector are preferred to identify with a high grade of certainty substances contained in complex biological matrices [13,14]. Analytical methodology involving chromatography coupled to mass spectrometry has been already developed for some of these compounds and their metabolites in murine urine and human plasma [15–24].

Within the framework of a Spanish survey carried out with consumers of classical psychostimulants and "hallucinogenic designer drugs" to gather information regarding the most consumed compounds, a liquid chromatography–electrospray ionization (ESI)-mass spectrometry method has been developed for identification and quantification of the most common misused "hallucinogenic designer drugs" in urine of consumers enrolled in the survey.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The standard of 3,4-methylendioxypropylamphetamine (MDPA used as internal standard, IS) was supplied by Salars (Como, Italy) and that of MDMA by Cerilliant (Austin, TX, USA).

Standards of 2,5-dimethoxy-4-methyl-phenethylamine (2C-D), 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B), 1-(8- Bromo-2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b'] difuran-4-yl)-2- aminoethane (2C-B-Fly), 4-ethylthio-2,5-dimethoxy-β-phenethylamine (2C-T-2), 4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I), and 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E), 1-(m-chlorophenyl)piperazine (m-CPP), 4-hydroxy-*N*,*N*-diisopropyltryptamine (4-OH-DIPT) and 4-acetoxy-*N*,*N*-diisopropyltryptamine (4-acetoxy-DIPT) were from Bravo Trading Ltd. (http://www.bravo-trading.com/Research_chemical.htm). Substances were sold as free pure substances. Bond Elut Certify solid-phase extraction (SPE) columns were from Varian (Palo Alto, CA, USA). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

### 2.2. Urine samples

Urine samples were from habitual consumers of designer drugs, which participated in a Spanish survey on the consumption of "hallucinogenic designer drugs" set up by the non-governmental organization Energy Control (www.energycontrol.org), which promotes harm reduction among drug users in Spain. All the individuals, which accepted to donate urine if and when they consumed any "hallucinogenic designer drugs" signed an informed consent. Samples were received by 26 men (age range 26–45 years) and 6 women (age range 23–26 years). Consumed drugs, as declared by the users, were the following: 2C-D, 2C-B, 2C-B in combination with MDMA, 2C-B-Fly, 2C-T-2, 2C-I, 2C-E, m-CPP, 4-OH-DIPT and 4-acetoxy-DIPT.

### 2.3. Instrumentation

LC–MS analyses were performed using an Agilent 1100 series HPLC system consisting on a G1312A binary pump, a G1322A degasser, and an ALS G1329A autosampler (Agilent Technologies, Palo Alto, CA) interfaced to an Agilent 1100 series G1946D mass spectrometer equipped with an atmospheric pressure ionization–electrospray ionization interface. Chromatographic separation was achieved using Thermo Electron-Hipersil Gold ultra pure silica column (150 mm × 4.6 mm; 5 μm) (CPS analitica, Milan, Italy). The mobile phase used in the separation, consisted of (A) 10 mM ammonium bicarbonate, pH 7.3 and (B) acetonitrile programmed as follows: initial 80% A for 3 min, decreased to 50% in 8 min, then increased again to 80% A in 9 min. The flow rate was set at 1 mL/min. All chromatographic solvents were degassed with helium before use. The injection volume was 20 μL and the column temperature was set at 27 °C.

The mass spectrometer was operated in positive ESI mode with selected ion monitoring (SIM) acquisition. The following ESI conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 12.0 L/min; nebulizer gas (nitrogen) at a pressure of 50 psi; capillary voltage at 4000 V.

MS characterization (purity and identity) of the compounds under investigation was achieved using direct infusion. The substances, dissolved in methanol at a concentration of 10 μg/mL, were infused through an integrated syringe pump into the ESI probe at a rate of 1 mL/min. In these experiments, full scan acquisitions were made over the (100–550 *m*/*z*) range in both negative and positive ionization. On the basis of experiments, the best acquisition parameters were also selected.

Three different fragmentation voltages (110, 150 and 200 V) were applied to obtain at least a quantifying ion for each particular analyte (the protonated molecule in the majority of the cases)

and two significant qualifying ions, accounting for 30–40% relative intensity. Dwell time was set at 15, 28 and 59 ms when the fragmentor voltages were set at 110, 150 and 200 V, respectively and mass peak width was 0.10 min.

More specifically, at 110 V ions at *m/z* 194, 163 and 105 for MDMA; 196, 179 and 164 for 2C-D; 222, 163 and 105 for MDPA; 242, 225 and 195 for 2C-T-2; 286, 269 and 188 for 2C-B-Fly and 308, 290 and 276 for 2C-I were selected. At 150 V ions at *m/z* 262, 245 and 164 for 2C-B and at 210, 193 and 178 for 2C-E were selected. Finally, at 200 V ions at *m/z* 261, 160 and 115 for 4-OH-DIPT; 303, 202 and 160 for 4-acetoxy-DIPT; 197, 154 and 119 for m-CPP were selected.

The $[M+H]^+$ ions at *m/z* 194 for MDMA, 196 for 2C-D, 222 for MDPA, 242 for 2C-T-2, 286 for 2C-B-Fly, 308 for 2C-I, 261 for 4-OH-DIPT, 303 for 4-acetoxy-DIPT, 197 for m-CPP and the fragment ions $[C_{10}H_{12}BrO_2]^+$ at *m/z* 245 for 2C-B and $[C_{12}H_{17}O_2]$ at *m/z* 193 for 2C-E were selected for quantification.

The acceptance criterion for selected ions intensity ratios was a deviation ≤20% of the average of the ion intensity ratios of all the calibrators.

### 2.4. Calibration standards and quality control samples

Stock standard solutions (100 μg/mL) of analytes were prepared in methanol. Working solutions at concentrations of 10 and 1 μg/mL were prepared by dilution of the stock standards with methanol. The IS working solution was used at a concentration of 10 μg/mL. Calibration standards containing analytes concentration at limit of quantification (LOQ), 100, 250, 750, 2000 and 4000 ng/mL were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 mL of pre-checked drug-free urine pool sample. Quality control (QC) samples of 3000 ng/mL (high), 500 ng/mL (medium), 75 ng/mL (low) and samples at LOQ of each analyte were prepared in drug-free urine, aliquoted and stored at −20 °C. They were included in each analytical batch to check calibration, accuracy and precision, and stability of samples under storage conditions.

### 2.5. Sample preparation

Sample preparation involved a solid-phase extraction procedure of both non-hydrolyzed and enzymatically hydrolyzed urine samples in order to assess also the presence of analytes under investigation as conjugated compounds.

Therefore, 1 mL urine spiked with 50 μL of IS working solution was transferred into 15 mL screw-capped tubes with 1 mL 1.0 M acetate buffer (pH 5.2) and 50 μL type H-2 β-glucuronidase from *Helix pomatia* (114,400 β-glucuronidase units/mL; 3290 sulphatase units/mL) were added. Hydrolysis

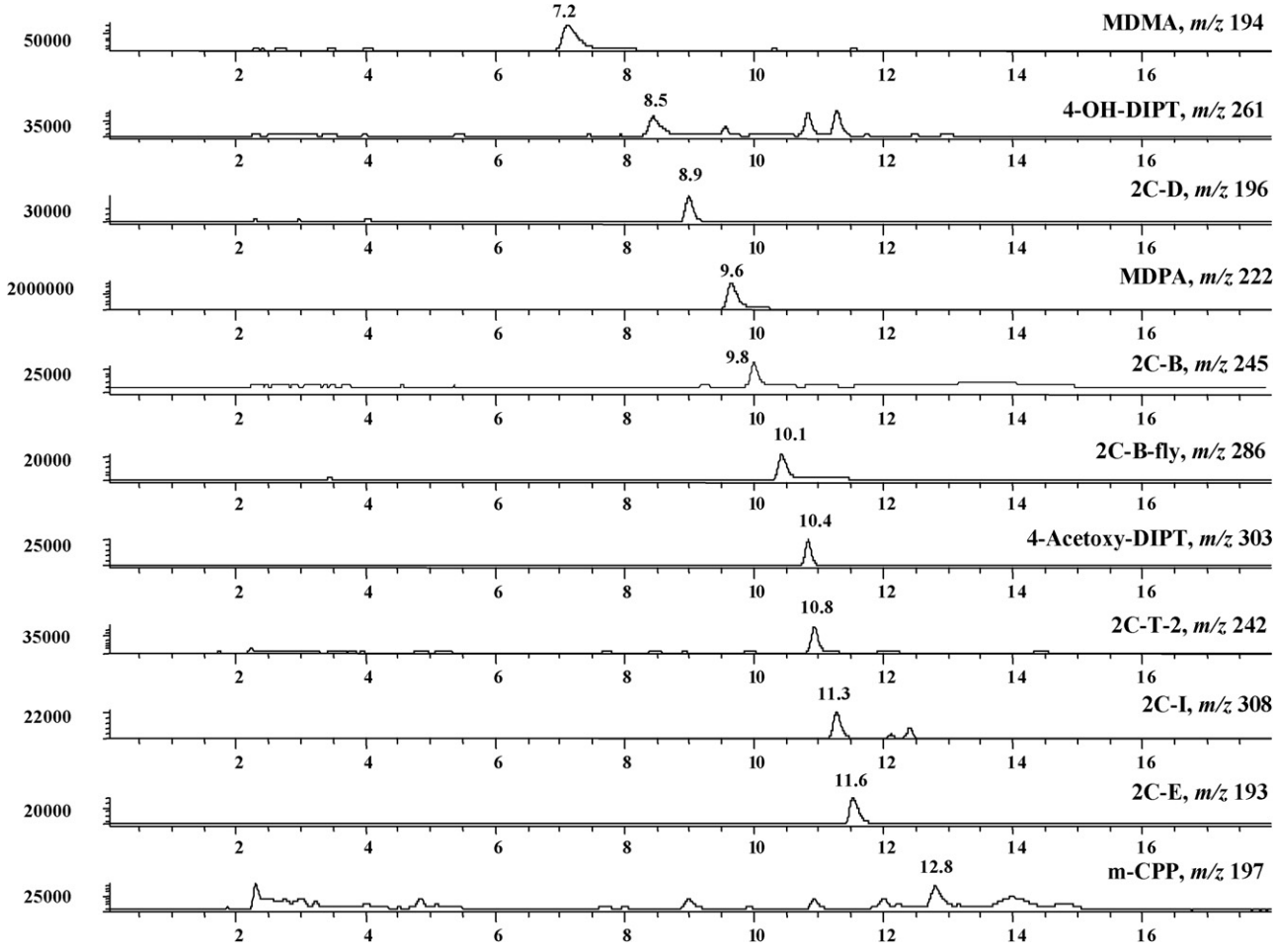


Fig. 1. SIM chromatogram of blank urine sample spiked with 75 ng/mL each of the compounds under investigation and 500 ng/mL IS.

was performed in a water bath for 16 h at 37 °C. Non-hydrolyzed urines were added with the same reagents as previously described, just before solid-phase extraction omitting the incubation step.

Urine samples added with 1 mL 0.1 M phosphate buffer pH 6.0 underwent solid-phase extraction procedure using Bond Elut Certify columns according to a previously reported method [25]. Columns were preconditioned with 2 mL methanol and 2 mL 0.1 M phosphate buffer pH 6.0, washed with 1 mL 1.0 M acetic acid and 4 mL methanol. Analytes were eluted with 2 mL ethyl acetate–2% ammonium hydroxide.

The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 100 μL 10 mM ammonium bicarbonate, pH 7.3. A 20 μL volume was injected into LC–MS system.

### 2.6. Validation procedures

Prior to application to real samples, the method was tested in a validation protocol following the accepted criteria for bioanalytical method validation [26,27]. Selectivity, matrix effect, recovery, linearity, limit of detection (LOD) and LOQ, precision, accuracy and stability were assayed.

#### 2.6.1. Selectivity

Twenty different drug-free urine samples from laboratory personnel were extracted and analyzed for assessment of potential interferences due to endogenous substances. The apparent responses at the retention times of the analytes under investigation and IS were compared to the response of analytes at the LOQ and IS at its lowest quantifiable concentration. Furthermore, potential interferences from principal psychoactive drugs and major metabolites such as opiates (6 monoacetylmorphine, morphine, codeine), cocaine and benzoylecogonine, cannabinoids ($\Delta^9$-tetrahydrocannabinol, 11-hydroxy-$\Delta^9$-tetrahydrocannabinol and 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid) benzodiazepines (clorazepate, diazepam, lorazepam, oxazepam, alprazolam, triazolam), antidepressants (imipramine, desipramine, clomipramine, desmethylclomipramine, amitriptyline, nortriptyline, fluoxetine, norfluoxetine, paroxetine) and amphetamine-type substances (amphetamine, methamphetamine, methyledioxyamphetamine, 3,4-methylenedioxyethylamphetamine, *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine)were also evaluated spiking 1 mL pre-checked drug-free urine pool with 2 μg of each compound and carried through the entire procedure.

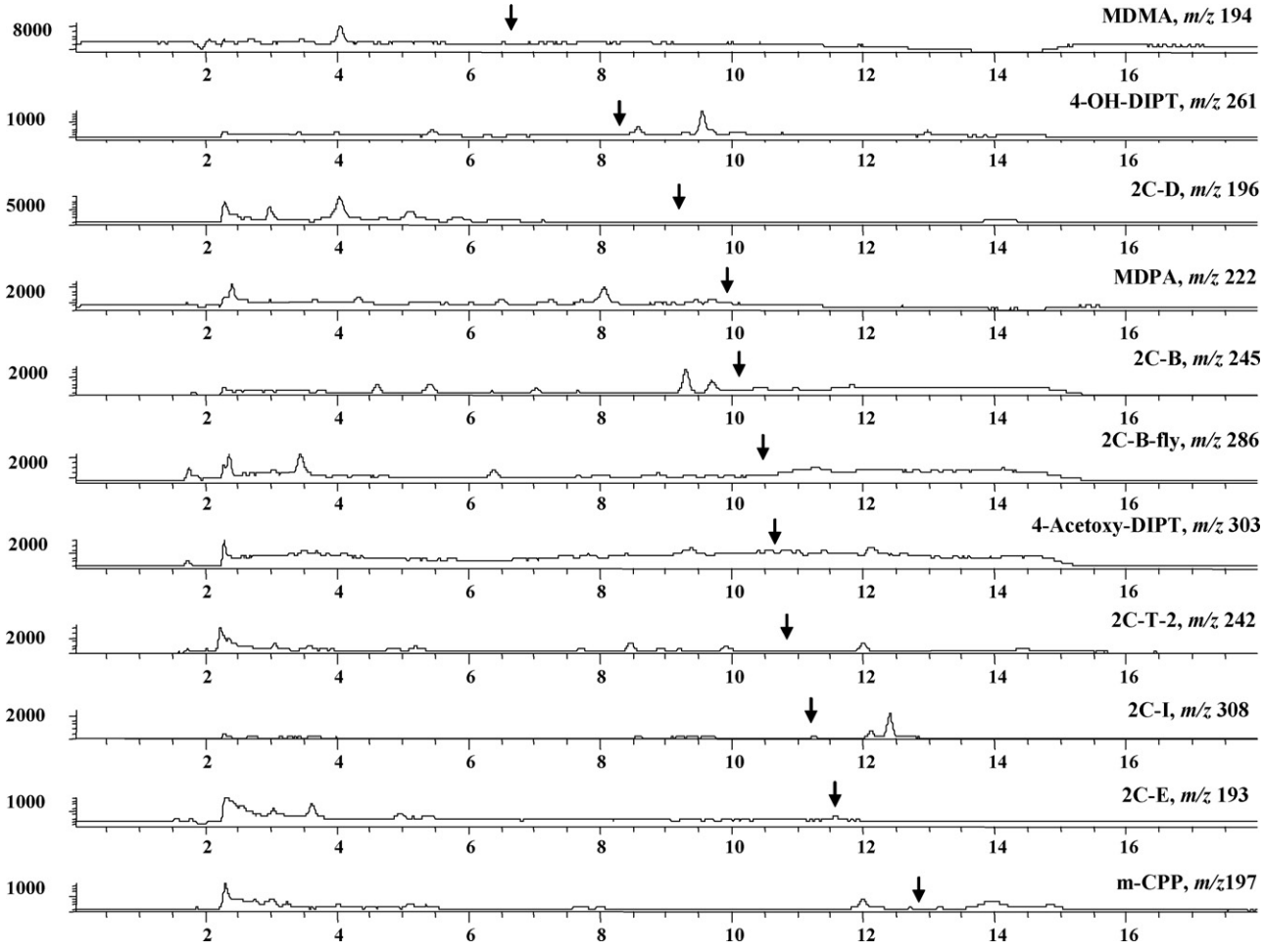


Fig. 2. SIM chromatogram of blank urine sample.

*2.6.2. Carryover*

The potential for carryover was investigated by injecting extracted drug-free urine samples with added IS, immediately after analysis of the highest concentration point of the calibration curve on each of the days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

*2.6.3. Recovery and matrix effect*

Analytical recoveries were calculated by comparing the peak areas obtained when QC samples were analyzed by adding the analytical reference standards and the IS in the extract of drug-free urine samples prior to and after the extraction procedure. The recoveries were assessed by QC samples using four replicates for each concentration level. For an evaluation of matrix effect, the peak areas of extracted drug-free samples spiked with standards at QC concentrations after the extraction procedure were compared to the peak areas of pure diluted substances.

*2.6.4. Calibration curves and linearity*

Calibration curves were tested over the LOQ 4000 ng/mL concentration range. Peak area ratios between compounds and IS were used for calculations. A weighted (1/concentration) least-squares regression analysis was used for slopes and intercepts. Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine the detection limit (LOD = 3 S.D.) and the quantification limit (LOQ = 10 S.D.). To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative S.D. and relative error, respectively.

*2.6.5. Accuracy and precision*

Five replicates at each of three different QC sample concentrations added to drug-free urine samples, extracted as reported above, were analyzed for the determination of intra-assay precision and accuracy. They were determined for three independent experimental assays. Precision was expressed as the relative S.D. (R.S.D.) of concentrations calculated for QC samples and accu-

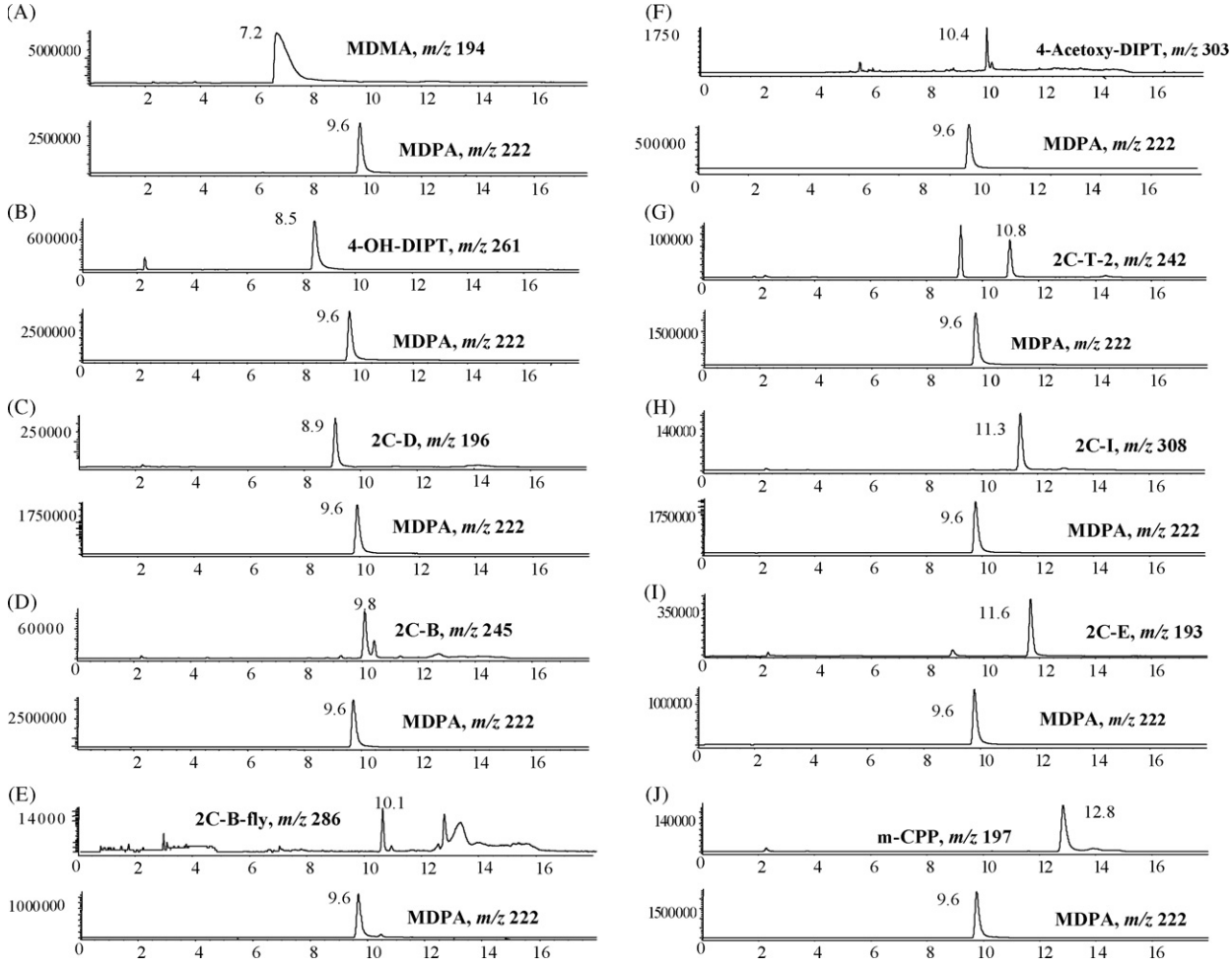


Fig. 3. (A) SIM chromatogram of an extracted sample **25** containing 6771.7 ng/mL MDMA. (B) SIM chromatogram of an extracted sample **42** containing 4630.8 ng/mL 4-OH-DIPT. (C) SIM chromatogram of an extracted sample **16** containing 123.9 ng/mL 2C-D. (D) SIM chromatogram of an extracted sample **18** containing 276.3 ng/mL 2C-B. (E) SIM chromatogram of an extracted sample **28** containing 28.6 ng/mL 2C-B-fly. (F) SIM chromatogram of an extracted sample **45** containing 87.5 ng/mL 4-Acetoxy-DIPT. (G) SIM chromatogram of an extracted sample **31** containing 355.1 ng/mL 2C-T-2. (H) SIM chromatogram of an extracted sample **32** containing 166.9 ng/mL 2C-I. (I) SIM chromatogram of an extracted sample **37** containing 77.1 ng/mL 2C-E. (J) SIM chromatogram of an extracted sample **39** containing 13959.3 ng/mL m-CPP.

racy as the relative error of the calculated concentrations. Both parameters had to be within 20% of R.S.D. or error. In addition, drug-free urine samples spiked with 8000 and 20,000, 40,000 and 80,000 ng/mL analytes under investigation were prepared as over-curve samples, to be tested for accuracy and precision once diluted 10 and 50 times, respectively.

#### 2.6.6. Freeze–thaw cycles and mid-term stability

The effects of three freeze–thaw cycles (storage at −20 °C) on the stability of compounds in urine were evaluated by repeated analysis ($n = 3$) of QC samples. In addition, mid-term stability test was performed for QC and real samples stored at −20 °C. Three replicates of both QC samples and one urine sample for each of the analytes under investigation were analyzed once a month during a 6 months period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes both in QC and real samples.

## 3. Results and discussion

### 3.1. Chromatography and validation results

A representative chromatogram obtained following the extraction of 1 mL urine sample spiked with analytes is shown in Fig. 1. Each chromatographic run was completed in 15 min, an analysis time necessary to elute and separate 11 compounds, in some case chemically heterogeneous. Samples following the ones exceeding the linear range in the chromatographic run were re-injected to check eventual contamination by carryover. Nonetheless, no carryover was observed in this case, nor when drug-free urine samples were injected after the highest point of the calibration curve. No additional peaks due to endogenous substances that could have interfered with the detection of compounds of interest were observed (Fig. 2). Similarly, none of the drugs of abuse or aforementioned medications, carried through the entire procedure, interfered with the assay.

With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted blank urine samples versus those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances.

Table 1
Recovery of "hallucinogenic designer drugs" under investigation

| Analyte | $n$ | Concentration (μg/L) | Mean recovery (%) | S.D. |
|---|---|---|---|---|
| 2C-D | 4 | 75 | 83.6 | 2.9 |
| | | 500 | 85.3 | 3.3 |
| | | 3000 | 82.6 | 1.3 |
| 2C-B | 4 | 75 | 85.2 | 5.3 |
| | | 500 | 80.9 | 2.6 |
| | | 3000 | 82.8 | 2.3 |
| 2C-B-Fly | 4 | 75 | 84.9 | 9.1 |
| | | 500 | 83.0 | 4.3 |
| | | 3000 | 82.9 | 0.9 |
| 2C-T-2 | 4 | 75 | 72.7 | 6.3 |
| | | 500 | 71.8 | 1.4 |
| | | 3000 | 73.5 | 4.1 |
| 2C-I | 4 | 75 | 76.7 | 0.9 |
| | | 500 | 74.5 | 1.4 |
| | | 3000 | 76.2 | 3.6 |
| 2C-E | 4 | 75 | 92.6 | 5.2 |
| | | 500 | 90.5 | 1.6 |
| | | 3000 | 91.2 | 8.4 |
| MDMA | 4 | 75 | 73.8 | 9.2 |
| | | 500 | 79.7 | 6.9 |
| | | 3000 | 75.8 | 4.1 |
| m-CPP | 4 | 75 | 95.6 | 9.6 |
| | | 500 | 91.8 | 3.1 |
| | | 3000 | 93.5 | 3.7 |
| 4-OH-DIPT | 4 | 75 | 59.3 | 4.5 |
| | | 500 | 55.4 | 2.9 |
| | | 3000 | 56.1 | 3.2 |
| 4-Acetoxy-DIPT | 4 | 75 | 75.3 | 1.1 |
| | | 500 | 72.7 | 1.9 |
| | | 3000 | 73.9 | 0.5 |

Absolute analytical recoveries (mean ± standard deviation, S.D.) obtained after extraction procedure for the three different QC samples were always higher than 70%, apart the 55–60% for the hydrophilic 4-OH-DIPT, with no relevant variations in extraction recovery at different concentration levels (Table 1).

Linear calibration curves showed determination coefficients ($r^2$) higher than 0.99 in all cases. LODs and LOQs values were

Table 2
Method calibration parameters

| Analyte | Slope[a] | Intercept[a] | Determination coefficient ($r^2$) | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|
| 2C-D | 0.00048 ± 0.00011 | 0.0004 ± 0.009 | 0.998 ± 0.001 | 16 | 53 |
| 2C-B | 0.00001 ± 0.000001 | −0.0004 ± 0.0003 | 0.994 ± 0.003 | 16 | 53 |
| 2C-B-Fly | 0.00014 ± 0.00001 | −0.004 ± 0.004 | 0.997 ± 0.002 | 16 | 53 |
| 2C-T-2 | 0.00016 ± 0.00001 | −0.008 ± 0.007 | 0.995 ± 0.004 | 8 | 27 |
| 2C-I | 0.00024 ± 0.00001 | −0.007 ± 0.004 | 0.998 ± 0.001 | 16 | 53 |
| 2C-E | 0.00266 ± 0.00058 | −0.012 ± 0.186 | 0.994 ± 0.003 | 16 | 53 |
| MDMA | 0.00049 ± 0.00003 | 0.084 ± 0.039 | 0.997 ± 0.006 | 16 | 53 |
| m-CPP | 0.00003 ± 0.000004 | 0.0001 ± 0.002 | 0.998 ± 0.007 | 16 | 53 |
| 4-OH-DIPT | 0.00006 ± 0.00001 | −0.001 ± 0.002 | 0.997 ± 0.002 | 16 | 53 |
| 4-Acetoxy-DIPT | 0.00024 ± 0.00016 | −0.006 ± 0.010 | 0.995 ± 0.002 | 11 | 36 |

[a] Mean and S.D. of five replicates.

Table 3
Intra-day ($n = 5$) and inter-day ($n = 15$) precision and accuracy

| Analyte | Intra-assay precision (R.S.D.) | | | Intra-assay accuracy (Error%) | | | Inter-assay precision (R.S.D.) | | | Inter-assay accuracy (Error%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 75 (ng/mL) | 500 | 1500 | 75 (ng/mL) | 500 | 1500 | 75 (ng/mL) | 500 | 1500 | 75 (ng/mL) | 500 | 1500 |
| 2C-D | 6.4 | 7.0 | 13.4 | 16.2 | 4.7 | 13.5 | 12.5 | 12.4 | 11.6 | 15.0 | 10.5 | 9.1 |
| 2C-B | 5.9 | 17.7 | 2.1 | 16.3 | 12.0 | 14.6 | 19.5 | 10.6 | 15.4 | 17.2 | 7.7 | 13.2 |
| 2C-B-Fly | 2.8 | 15.1 | 4.7 | 2.5 | 10.4 | 3.4 | 18.0 | 11.5 | 17.4 | 13.3 | 9.4 | 13.6 |
| 2C-T-2 | 8.7 | 10.5 | 8.5 | 18.3 | 10.6 | 6.3 | 19.8 | 15.0 | 11.0 | 20.0 | 12.1 | 9.0 |
| 2C-I | 0.4 | 4.6 | 18.0 | 10.4 | 10.8 | 14.1 | 9.4 | 16.5 | 14.6 | 9.7 | 13.6 | 12.1 |
| 2C-E | 19.2 | 14.1 | 3.4 | 18.6 | 15.0 | 10.3 | 19.3 | 17.1 | 10.1 | 16.0 | 14.6 | 9.0 |
| MDMA | 19.9 | 6.4 | 1.1 | 16.9 | 5.6 | 12 | 14.1 | 11.2 | 16.2 | 12.9 | 8.4 | 13.8 |
| m-CPP | 3.5 | 12.4 | 10.1 | 12.9 | 15.1 | 15.7 | 10.4 | 19.1 | 19.0 | 17.3 | 15.4 | 17.6 |
| 4-OH-DIPT | 19.1 | 10.6 | 9.7 | 13.7 | 7.6 | 7.3 | 11.2 | 11.0 | 7.8 | 8.5 | 8.5 | 6.1 |
| 4-Acetoxy-DIPT | 16.8 | 8.6 | 8.9 | 15.3 | 12.6 | 7.3 | 16.2 | 9.8 | 10.2 | 13.9 | 11.1 | 9.5 |

and adequate for the purposes of the present study (Table 2). Particularly, the calculated LOQ tested for precision and accuracy presented coefficient of variations always better that 20%.

The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria (Table 3) [26,27]. Over-curve samples, tested for accuracy and precision after diluting 10 and 50 times,

Table 4
Analyte concentrations (ng/mL) in non-hydrolyzed and enzymatically hydrolyzed urine samples from consumers

| No. | Substance | Time (h) of urine collection | Concentration without hydrolysis | Concentration after hydrolysis (% conjugation) |
|---|---|---|---|---|
| 15 | 2CD | 2.50 | 81.3 | 107.1 (24%) |
| 16 | 2CD | 2.50 | 123.9 | 302.7 (59%) |
| 17 | 2C-B | 2.33 | 1656.3 | 3039.6 (46%) |
| 18 | 2C-B | 2.00 | 276.3 | 822.4 (66%) |
| 19 | 2C-B | 2.00 | 2254.2 | 23791.8 (91%) |
| 20 | 2C-B | 2.25 | 1099.5 | 9618.0 (89%) |
| 21 | 2C-B | 2.25 | 2417.0 | 28060.8 (91%) |
| 22 | 2C-B | 2.17 | 4912.8 | 59413.1 (92%) |
| 23 | 2C-B | 4.00 | 4892.6 | 31932.5 (85%) |
| 24 | MDMA + 2C-B | 2.00 | 6771.7–27090.9 | 25400.7–34710.1 (73%–22%) |
| 25 | MDMA + 2C-B | 3.00 + 2.00 | 774.5–7259.2 | 1620.5–7249.6 (52%–0%) |
| 26 | MDMA + 2C-B | 2.00 | 0.0–11910.9 | 0.0–17361.1 (– – 31%) |
| 27 | MDMA + 2C-B | 3.00 | 15189.7–33606.4 | 15472.6–33626.2 (1.7%-0%) |
| 28 | 2C-B-FLY | 3.00 | 28.6[a] | 102.9 (72%) |
| 29 | 2C-T-2 | 2.50 | 28.5 | 236.9 (89%) |
| 30 | 2C-T-2 | 2.00 | 133.2 | 431.7 (69%) |
| 31 | 2C-T-2 | 2.50 | 355.1 | 545.3 (35%) |
| 32 | 2C-I | 2.00 | 166.9 | 1429.5 (88% |
| 33 | 2C-I | 2.00 | 121.4 | 561.5 (78%) |
| 34 | 2C-I | 3.75 | 0.0 | 851.5 (100%) |
| 35 | 2C-I | | 82.2 | 1096.1 (93%) |
| 36 | 2C-I | | 66.1 | 249.4 (74%) |
| 37 | 2-C-E | 2.50 | 77.1 | 258.5 (70%) |
| 38 | 2-C-E | 2.00 | 0.0 | 68.4 (100%) |
| 39 | m-CPP | 3.00 | 13959.3 | 20384.4 (32%) |
| 40 | 4-HO-DIPT | 2.08 | 1304.8 | 5776.8 (77%) |
| 41 | 4-HO-DIPT | 2.50 | 1331.4 | 45345.1 (97%) |
| 42 | 4-HO-DIPT | 3.00 | 4630.8 | 59802.3 (92%) |
| 43 | 4-Acetoxy-DIPT | 2.00 | 0.0 | 0.00 (–) |
| 44 | 4-Acetoxy-DIPT | 2.00 | 0.0 | 0.00 (–) |
| 45 | 4-Acetoxy-DIPT | 3.00 | 87.5 | 87.4 (0%) |
| 46 | 4-Acetoxy-DIPT | 3.00 | 88.8 | 84.1 (0%) |

[a] Concentration value obtained by analysis of 2 mL urine sample.

gave values always better than 10% relative standard deviation (R.S.D.) and error %.

No relevant degradation was observed after any of the three freeze/thaw cycles, with differences in the initial concentration less than 10%. Similar results (differences to the initial concentration always lower than 10%) were obtained in the case of the mid-term stability test for both QC and real samples assuring the feasibility of stored samples analysis.

### 3.2. Analysis of urine samples

The validated assay has been applied to urine samples from consumers of "hallucinogenic designer drugs" before and after enzymatic cleavage of conjugates (Table 4, Fig. 3).

From the results obtained, it can be said that phenetylamine compounds (2 C-B, 2 C-E, etc.) can be found in urine mainly as sulphate and glucuronide conjugates. Interestingly, in case of co-administration of MDMA and 2 C-B, the portion of this latter drug measured as conjugate (from 0 to 31%) appears less than that measured when 2C-B is consumed alone (from 46 to 92%). A metabolic interaction between MDAM and 2 C-B may be hypothesized, as both substances are substrates of monoamine oxidases (MAOs) A and B [28,29], but at present this observation is quite speculative, given the small number of analyzed samples, and further studies are required to confirm the given hypothesis.

Similarly to phenetylamines, 4-OH-DIPT appeared mainly as conjugated compound in urine samples. Concerning 4-acetoxy-DIPT, the substance was found as unconjugated compound in only two of the four urine samples from subjects which declared substance consumption. The possible conversion to 4-OH-DIPT was investigated, but no trace of this latter compound was found in samples under analysis. On the other hand, since the product consumed by the four subjects who donated urine samples was examined and identified in all cases as 4-acetoxy-DIPT, there was no reason to believe that two of them consumed substance other than this one. Most probably, 4-acetoxy-DIPT underwent an extensive metabolism and was excreted as unaltered substance in very low amount.

Unfortunately, a limitation of the present study was that the analytical methodology was developed only for identification and quantification of parent compounds in urine samples, leaving out any investigation concerning metabolites, which would have been of help especially in this concern.

However, since the aim of the study was the development and validation of an analytical assay to detect "hallucinogenic designer drugs" for clinical and forensic toxicology purposes, urine samples were collected few hours after the drug intake, trying to mimic a situation occurring after an eventual acute intoxication when subject is referred to the Emergency Rooms of a hospital.

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