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A simple and reliable procedure for the determination of psychoactive drugs in oral fluid by gas chromatography–mass spectrometry

Mitona Pujadas [a,1], Simona Pichini [b], Ester Civit [a,1], Elena Santamariña [c,1], Katherine Perez [c,1], Rafael de la Torre [a,d,*,1]

[a] *Grup de Recerca Clínica en Farmacologia Humana i Neurociencies, Unitat de Farmacologia, Institut Municipal d'Investigació Mèdica, Dr. Aiguader 88, 08003 Barcelona, Spain*
[b] *Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy*
[c] *Agencia de Salut Publica de Barcelona, Barcelona, Spain*
[d] *Universitat Pompeu Fabra, Dr. Aiguader 88, 08003 Barcelona, Spain*

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Abstract

A simple and reliable gas chromatography–mass spectrometry method for identifying and quantifying psychoactive drugs in oral fluid is described. Substances under investigation were: psychostimulant drugs (amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxiamphetamine, 3,4-methylenedioxy-*N*-ethylamphetamine, phentermine), cocaine and metabolites (benzoylecgonine, cocaethylene, and ecgonine methyl esther), cannabinoids (delta-9-tetrahydrocannabinol, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol, 11-hydroxy-delta-9-tetrahydrocannabinol, cannabinol and cannabidiol), opiates (6-monoacetylmorphine, morphine and codeine), hypnotics (flurazepam, flunitrazepam, dipotassium chlorazepate, alprazolam, diazepam and oxazepam), antidepressant drugs (amitryptiline, paroxetine and sertraline), antipsychotic drugs (haloperidol, chlorpromazine and fluphenazine) chlormethiazole, loratidine, hydroxyzine, diphenhydramine, valproic acid and gabapentin. After the addition of deuterated analogues of morphine, 3,4-methylenedioxymethamphetamine, (±)-11-nor-9-carboxy-delta-9-tetrahydrocannabinol and clonazepam as internal standards, all the compounds were simultaneously extracted from oral fluid by solid-phase extraction procedure. Acid compounds were eluted with acetone while basic and neutral compounds with dichloromethane:isopropanol:ammonium (80:20:2, v/v/v). Chromatography was performed on a methylsilicone capillary column and analytes, derivatized with *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide, were determined in the selected-ion-monitoring (SIM) mode. Mean recovery ranged between 44.5 and 97.7 % and quantification limit between 0.9 and 44.2 ng/ml oral fluid for the different analytes. The developed analytical methodology was applied to investigate the presence of psychoactive drugs in oral fluid from injured individuals attending the emergency room (MACIUS project).

Keywords: Psychoactive drugs; Oral fluid; Gas chromatography–mass spectrometry

1. Introduction

Biological matrices alternative to urine and plasma have recently been introduced for assessing drug exposure [1]. Oral fluid (saliva), sweat and hair are alternative biologic matrices, which have been extensively and successfully used to assess recent and past and/or acute and chronic exposure to drugs of abuse.

Oral fluid is the only fluid that has been successfully used as an alternative to blood in several pharmacokinetic and pharmacotoxicologic studies including drugs of abuse [2–4] and there is evidence that when a given drug is dectected in oral fluid specimens, there is a high likelihood for a subject being under the pharmacologic effects of the drug [5]. In addition, there are a number of reports suggesting that oral fluid

* Corresponding author at: Grup de Recerca Clínica en Farmacologia Humana i Neurociencies, Unitat de Farmacologia, Institut Municipal d'Investigació Mèdica, Dr. Aiguader 88, 08003 Barcelona, Spain. Tel.: +34 93 3161484; fax: +34 93 3160410.
E-mail address: rtorre@imim.es (R. de la Torre).
[1] Investigators of the MACIUS Project.

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could also be an alternative to urine for drugs of abuse testing [6,7].

The physiology of oral fluid as well as the mechanisms of drug transfer of drugs into saliva have been recently reviewed [8]. The advantage of oral fluid over traditional matrices like urine and blood is that collection is almost non-invasive and relatively easy to perform. Supervision of sample collection can be achieved without annoying subjects providing it [9]. Some disadvantages, however, are related to oral contamination from certain routes of administration (smoking, snorting, oral ingestion) and to the method of sample collection that may influence oral fluid drug concentrations as a result of changes in pH and flow rate [10]. Previous studies on drugs detection in oral fluid have shown that weak bases, such as MDMA, cocaine, opiates, benzodiazepines, or nicotine tend to concentrate in this matrix because its pH is slightly more acidic than of plasma [4,11]. Although some metabolites have been detected, the parent drug is usually the main analyte found.

Oral fluid flow can be stimulated to ensure adequate sample volume. Nonetheless, the use of specific devices that stimulate fluid production usually reduce drug concentration with respect to a non-stimulated collection (e.g. spiting), because fluid stimulation modifies the pH gradient between this fluid and plasma (the pH of oral fluid becomes more alkaline) and thus drug diffusion is reduced [11,12]. Another aspect to be considered is the recovery of drugs from collection devices that may depend on their components, but also on how oral fluid is preserved and stored until analysis [13].

When developing an analytical methodology for the detection of drugs in oral fluid it has to take into account the limited amount of sample available (1–3 ml) and sensitivity requirements as concentrations are higher or similar than those found in plasma but at least one order of magnitude lower than urinary ones considering also that analytes are not the same in both biological specimens. These limitations apply to research/work cases of driving under the influence of drugs (DUID) and testing for them in oral fluid. Despite a reasonable good correlation between oral fluid concentrations and those encountered in plasma on one hand and the impairment in psychomotor performance induced by drugs on the other, there are some challenges to be faced in the areas of the sensitivity and reliability of analytical methodologies applied in drug testing [14]. Several authors postulate that mass spectrometry coupled to chromatographic techniques offer a more flexible, specific and sensitive alternative to the screening of oral fluid samples for drugs of abuse than immunoassays [15].

Indeed, in recent years different methods involving both gas and liquid chromatography coupled to mass spectrometry have been reported, which determined different classes of illicit drugs and psychoactive pharmaceuticals (e.g. benzodiazepines) in oral fluid [16–25].

It has to be said that methodologies involving mass spectrometry as detector are preferred to identify with a high degree of sensitivity, selectivity and certainty substances contained in oral fluid. Whereas a standard gas chromatograph–mass spectrometer is an apparatus generally found in analytical laboratories and easy to use, the same is not with liquid chromatographs coupled to mass spectrometry or tandem mass spectrometry. Furthermore, the simultaneous detection of different classes of substances has required lengthy extraction procedures, solid phase extractions or more than three different steps, finally appearing complex and time-consuming.

The MACIUS project was designed to estimate the prevalence of psychoactive drugs among persons injured by any mechanism who attended an emergency room for medical care within the 6 h posterior to the injury. Within the framework of the MACIUS project, we developed and validated a simple and reliable assay to simultaneously identify 36 psychoactive drugs and quantify 30 of them, candidate to be present in oral fluid by gas chromatography coupled to mass spectrometry (GC/MS).

2. Experimental

2.1. Chemicals and materials

Amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), cocaine, benzoylecgonine, cocaethylene, ecgonine methyl ester, 6-monoacetyl-morphine (6-MAM), codeine, delta-9-tetrahydrocannabinol (Δ-9-THC), 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (Δ-9-THC-COOH), 11-hydroxy-delta-9-tetrahydrocannabinol (Δ-9-THC-OH), cannabinol, cannabidiol, flunitrazepam, alprazolam, diazepam, oxazepam, clonazepam, amitriptyline, sertraline, dipotassium clorazepate, chlorpromazine, **d_3**-Δ-9-THC-COOH and **d_5**-MDMA were supplied by Cerilliant (Austin, TX, USA). 3,4-methylenedioxiamphetamine (MDA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), morphine and **d_3**-morphine were purchased from Lipomed Inc. (Cambridge, MA, USA). Paroxetine was obtained through GlaxoSmithKline (TresCantos, Madrid, Spain). Methamphetamine, haloperidol, fluphenazine, diphenhydramine, valproic acid, hydroxyzine, gabapentin, loratadine and chlormethiazole were from Sigma–Aldrich Corporation (St. Louis, MO, USA). Phentermine was from Pfizer (New York, NY, USA).

Bond Elut Certify® solid-phase extraction (SPE) columns were obtained from Varian Corp. (Harbor City, CA, USA). Gas chromatography grade *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). Ultra pure water was obtained using a Milli-Q purification system (Milli-pore, Molsheim, France). All other reagent grade chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions

Separate stock solutions (1 mg/ml) of all substances tested were prepared in HPLC-grade methanol and stored at −20 °C. From stock solutions, working solutions of 10 and 1 μg/ml were made and used for the preparation of calibration curves. Internal standards (ISTDs) (d_5-MDMA used as internal standard for amphetamine, MDA, methamphetamine, MDEA and MDMA, d_3-Δ-9-THC-COOH used as internal standard for Δ-9-THC, Δ-9-THC-COOH, Δ-9-THC-OH, cannabinol, and cannabidiol, d_3-morphine used for all the other analytes under investigation) were opportunely diluted in methanol to give a working solution

of 1 μg/ml stored at −20 °C until use oxazepam, flunitrazepam, valproic acid, alprazolam, chlormethiazole and paroxetine were excluded from the final method validation protocol, since poor validation parameters (recovery, intra and interassay variability) were obtained in the preliminary experiments. Nonetheless, stock and working solutions of these substances were prepared and included in the chromatographic–mass spectrometric process to obtain at least a qualitative (or semiquantitative) result in case of real samples.

Daily calibration curves were obtained by analyzing pre-checked blank oral fluid pooled samples spiked with quantification limit, 50, 100, 150 and 200 ng of each drug per ml oral fluid. Quality control (QC) samples (30, 75, 125 ng/ml oral fluid) were included in each analytical batch for calculation of validation parameters. Calibration and quality control samples were treated and processed as unknown samples.

2.3. Oral fluid samples

Samples analyzed in the present study were obtained from adults injured, who attended an emergency room of eight general university hospitals in Catalonia, Spain. They were asked to provide an oral fluid sample on voluntary basis in addition to a hair sample. The study protocol was approved by the Local Ethics Committees of participating hospitals and a signed consent was obtained from all participating individuals.

Oral fluid samples were collected at each different Hospital by chewing the Salivette® device (neutral cotton wool swab, Sarstedt, Germany) for 2 min. Once obtained, the device was transferred in a plastic tube, added with 1 ml mixture 1 M potassium phosphate pH 7.4, 150 mM NaCl, 0. 02% thimerosal, and stored at 4 °C. Within the following 12 h, tubes were sent to the analytical laboratory where they were centrifuged at 1200 rpm for 4 min. Supernatant was transferred in a criotube and stored at −20 °C prior to analysis. Then, the Salivette® collection device inside the tube was wetted with 2 ml methanol to extract cannabinoid type compounds, centrifuged at 1200 rpm for 4 min and recovered methanol extract was stored at −20 °C prior analysis.

2.4. Sample preparation and extraction

Methanol extracts were evaporated to dryness under nitrogen stream at 23 °C (c.a. 10 psi pressure) and reconstituted with 1 ml thawed oral fluid buffered supernatant (corresponding to the methanol extract) and with 1 ml 0.1 M sodium phosphate buffer (pH 6). The combination of oral fluid and its corresponding dried methanolic extract is defined as "sample".

Samples, calibration and QC samples were added with 50 μl of 1 μg/ml $\mathbf{d_5}$-MDMA, $\mathbf{d_3}$-morphine, $\mathbf{d_3}$-Δ-9-THC-COOH as ISTDs. Reconstituted extracts underwent a SPE procedure with Bond Elut Certify columns.

A previously reported extraction protocol [5] was applied with minor differences; 2 ml methanol and 2 ml phosphate buffer (pH 6) were used for conditioning the columns, followed by sample application at 1 ml/min, rinsing with 2 ml 0.25 M acetic acid and drying by applying a negative pressure (vacuum) to the column outlet for 5 min. Acidic compounds were eluted with 2 ml acetone at 1 ml/min. These extracts were evaporated under nitrogen stream at 50 °C (c.a. 15 psi pressure).

After the elution of acidic compounds, columns were rinsed with 2 ml methanol at 1.5 ml/min and 2 ml freshly prepared solution of dichloromethane:isopropanol:ammonium (80:20:2, v/v/v) were used at 1 ml/min to elute alkaline and neutral analytes. The eluates were collected using the tubes containing the dried extracts of acidic compounds. These mixed eluates were added with 20 μl MSTFA to prevent amphetamines losses and evaporated to dryness under nitrogen stream at 40 °C (c.a. 10 psi pressure). Trimethylsilyl derivatives were formed by reaction with 50 μl MSTFA as derivatization agent in a dry bath at 100 °C for 30 min.

2.5. GC–MS conditions

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973 N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation). Samples were injected in splitless mode and analytes separation was achieved on a methylsilicone capillary column (Ultra 1, 16.5 m × 0.2 mm i.d., 0.11 μm film thickness, Agilent Technologies). The oven temperature was programmed at 70 °C (2 min), followed by a 30 °C/min ramp to 160 °C, 5 °C/min to 170 °C, 20 °C/min to 200 °C, 10 °C/min to 220 °C and finally increased 30 °C/min ramp to 300 °C. The injector and the interface were operated at 280 °C. Helium was used as carrier gas at a flow rate of 0.8 ml/min.

The mass spectrometer was operated in electron impact ionization mode at 70 eV. The electron-impact (EI) mass spectra of the analyte and ISTDs were recorded in scan mode (scan range 40–550 *m*/*z*) to determine retention times and characteristic mass fragments. For routine analysis, three characteristic mass fragments were monitored in the selected-ion-monitoring (SIM) mode. Ions selected for substances identification and quantification are shown in Table 1. Ion ratio acceptance criterion was a deviation ≤20% ion ratios mean from all the calibration samples.

2.6. Validation procedures

Prior to application to real samples, the method was tested in a 4-day validation protocol. Selectivity, recovery, matrix effect, linearity, precision, accuracy, freeze-thaw cycles and limits of detection (LOD) and quantification (LOQ), were assayed.

Twenty different oral fluid samples were extracted and analyzed for assessment of potential interferences due to substances other than analytes under investigation. The apparent responses at the retention times of different analytes and ISTDs were compared to the response of analytes at the LOQ and ISTDs at its lowest quantifiable concentration. The potential for carryover was investigated by injecting extracted drug-free oral fluid samples, with added ISTDs, immediately after analysis of the

Table 1
m/z ions selected for substances identification and quantification

Compound	SIM ions	Retention time (min)
Valproic acid-*O*-TMS[a]	*m/z* 201, 174, 145	3.46
Amphetamine-*N*-TMS	*m/z* 116, 91, 192	4.40
Methamphetamine-*N*-TMS	*m/z* 130, 91, 206	4.81
Phentermine-*N*-TMS	*m/z* 114, 130, 206	4.84
Ecgonine methyl ester-*O*-TMS	*m/z* 96, 182, 271	5.84
Gabapentin-bis-*O,N*-TMS	*m/z* 210, 225, 182	5.84
MDA-*N*-TMS	*m/z* 116, 100, 236	6.20
d_5-MDMA-*N*-TMS	*m/z* 134, 255, 104	6.74
MDMA-*N*-TMS	*m/z* 130, 250, 100	6.78
MDEA-*N*-TMS	*m/z* 144, 135, 264	7.55
Diphenhydramine	*m/z* 165, 58, 152	8.01
Cocaine	*m/z* 182, 303, 272	10.26
Amitryptiline	*m/z* 58, 202, 215	10.28
Cocaethylene	*m/z* 196, 317, 82	10.66
Clorazepate	*m/z* 341, 327, 227	10.71
Chlormethiazole[a]	*m/z* 117, 313, 132	10.71
Benzoylecgonine-*O*-TMS	*m/z* 240, 82, 361	10.81
Cannabidiol-bis-*O*-TMS	*m/z* 390, 337, 301	10.90
Δ-9-THC-*O*-TMS	*m/z* 371, 386, 315	11.40
Oxazepam-bis-*O,N*-TMS[a]	*m/z* 429, 313, 340	11.54
Diazepam	*m/z* 283, 256, 221	11.62
Codeine-*O*-TMS	*m/z* 371, 178, 313	11.76
Cannabinol-*O*-TMS	*m/z* 367, 382, 310	11.79
Chlorpromazine	*m/z* 318, 272, 86	11.92
d_3-Morphine-bis-*O*-TMS	*m/z* 432, 417, 404	12.08
Morphine-bis-*O*-TMS	*m/z* 429, 414, 401	12.08
Sertraline-*N*-TMS	*m/z* 274, 348, 334	12.16
6-MAM-*O*-TMS	*m/z* 399, 340, 287	12.25
Flunitrazepam[a]	*m/z* 285, 312, 266	12.33
Paroxetine-*N*-TMS[a]	*m/z* 116, 249, 401	12.49
Δ-9-THC-OH-bis-*O*-TMS	*m/z* 371, 474, 459	12.50
d_3-Δ-9-THC-COOH-bis-*O*-TMS	*m/z* 374, 476, 491	12.93
Δ-9-THC-COOH-bis-*O*-TMS	*m/z* 371, 473, 488	12.93
Flurazepam	*m/z* 86, 387, 99	12.96
Alprazolam-*O*-TMS[a]	*m/z* 308, 279, 273	13.50
Hydroxyzine-*O*-TMS	*m/z* 201, 299, 446	13.54
Haloperidol-*O*-TMS	*m/z* 296, 206, 429	13.77
Fluphenazine-*O*-TMS	*m/z* 280, 406, 509	13.92
Loratadine	*m/z* 382, 266, 245	13.92

The underlined ions were selected for the quantification measurement.
[a] These compounds were not included in method validation, but could be identified by the developed methodology.

highest concentration point of the calibration curve on each of the 4 days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

Absolute analytical recoveries were calculated by comparing the peak areas obtained when QC samples were analyzed by adding the analytical reference standards and the ISTDs in the extract of drug-free oral fluid samples prior to and after the extraction procedure. The recoveries were assessed at three concentration levels, using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted drug-free oral fluid samples spiked with standards at a mean concentration level (75 ng/ml) after the extraction procedure were compared to the peak areas of pure diluted substances.

Calibration curves ($n = 4$) were tested over the working concentration range for all the compounds under investigation. Peak area ratios between compounds and I.S. were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (Statistical Package for the Social Sciences SPSS, version 12.0 for Microsoft Windows, Microsoft Corp., Seattle, USA). Ten replicates of drug-free oral fluid samples were used for calculating the limit of quantification. 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 = 3.3 S.D.) and quantification limit (LOQ = 10 S.D.). Once calculated, LOQ was tested for accuracy and precision to meet the established international criteria [26,27].

A total of five replicates at each of three quality control concentrations were added to drug-free oral fluid samples which were extracted, as reported above, and analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Inter-assay precision was expressed as the relative S.D. (R.S.D.) of concentrations calculated for quality control samples. Inter-assay accuracy was expressed as the relative error of the calculated concentrations.

The effect of three freeze–thaw cycles (storage at −20 °C) on compounds stability was evaluated on quality control samples in triplicate. The stability was expressed as a percentage of the initial concentration of the analytes spiked in drug-free oral fluid samples and quantified just after preparation.

3. Results and discussion

3.1. GC–MS

Representative chromatograms obtained following the extraction of: drug-free oral fluid (A), drug-free oral fluid samples spiked with all the analytes under the investigation (B) and a real sample (C) are shown in Fig. 1. When analytes concentrations in samples resulted higher than those in the calibration curve range, a smaller amount of samples was re-extracted and analyzed following standard procedure. Samples following the one exceeding the linear range in the chromatographic run were re-injected to check eventual contamination by carryover. Nonetheless, neither in this case any carryover was observed, nor when drug-free oral fluid samples were injected after the highest point of the calibration curve. The chromatographic separation of all compounds tested was achieved in 14.5 min. No additional peaks due to substances in drug-free samples that could have interfered with the detection of compounds of interest were observed. With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted drug-free samples versus those for pure diluted standards showed less than 10% analytical signal suppression. Psychoactive substances selected to be screened for take into account those more prevalent in Spain. Nevertheless, the methodology developed can incorporate additional substances. Several compounds which could be detected following the analytical method developed (oxazepam, flunitrazepam, valproic acid, alprazolam, paroxetine and chlormethiazole)

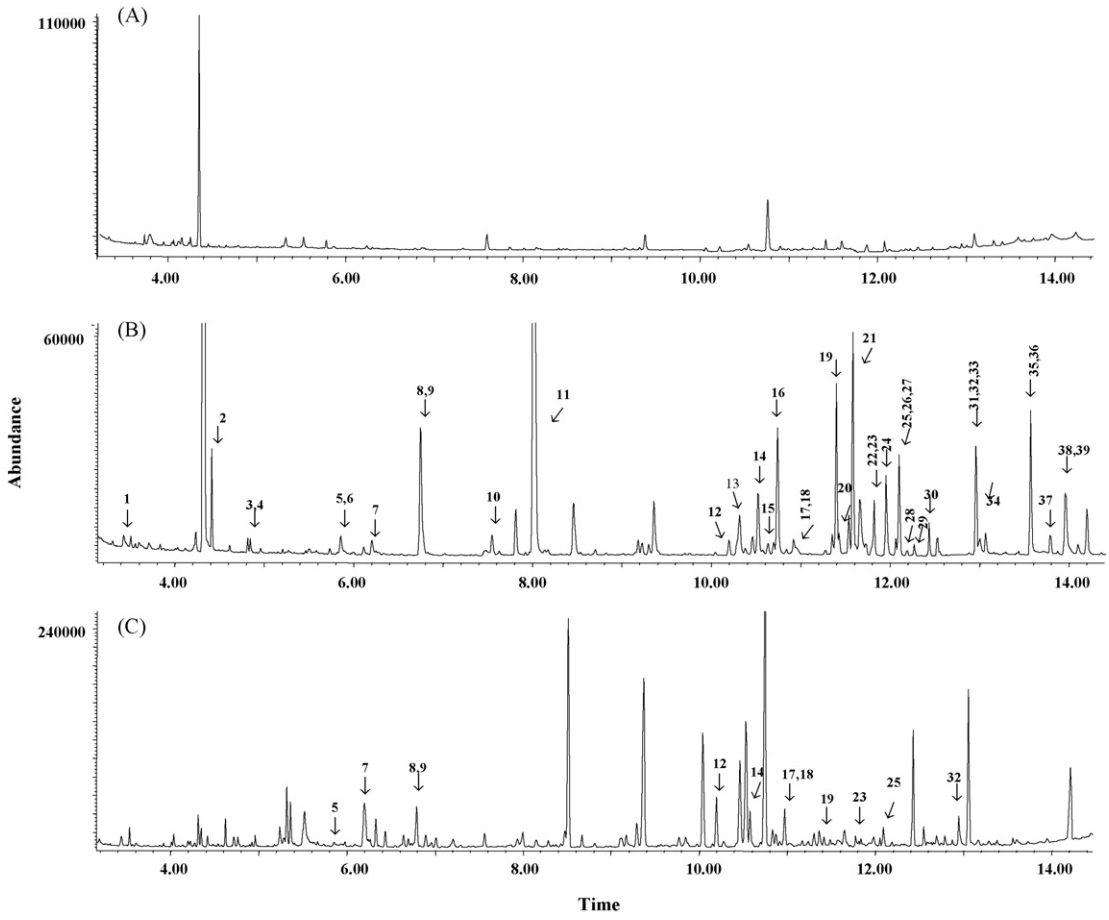

Fig. 1. Total ion current chromatograms obtained following the extraction of: (A) a drug-free oral fluid; (B) a drug-free oral fluid sample spiked with analytes under the investigation at a concentration of 50 ng/ml (1 = Valproic acid-*O*-TMS; 2 = Amphetamine-*N*-TMS; 3 = Methamphetamine-*N*-TMS; 4 = Phentermine-*N*-TMS; 5 = Ecgonine methylester-*O*-TMS; 6 = Gabapentin-bis-*O,N*-TMS; 7 = MDA-*N*-TMS, 8 = d_5-MDMA-*N*-TMS; 9 = MDMA-*N*-TMS; 10 = MDEA-*N*-TMS; 11 = Diphenhydramine; 12 = Cocaine; 13 = Amitryptiline; 14 = Cocaethylene; 15 = Clorazepate; 16 = Chlormethiazole; 17 = Benzoylecgonine-*O*-TMS; 18 = Cannabidiol-bis-*O*-TMS; 19 = Δ-9-THC-*O*-TMS; 20 = Oxazepam-bis-*O,N*-TMS; 21 = Diazepam; 22 = Codeine-*O*-TMS; 23 = Cannabinol-*O*-TMS; 24 = Chlorpromazine; 25 = d_3-Morphine-bis-*O*-TMS; 26 = Morphine-bis-*O*-TMS; 27 = Sertraline-*N*-TMS; 28 = 6-MAM-*O*-TMS; 29 = Flunitrazepam; 30 = Paroxetine-*N*-TMS; 31 = Δ-9-THC-OH-bis-*O*-TMS; 32 = d_3-Δ-9-THC-COOH-bis-*O*-TMS; 33 = Δ9-THC-COOH-bis-*O*-TMS; 34 = Flurazepam; 35 = Alprazolam-*O*-TMS; 36 = Hydroxyzine-*O*-TMS; 37 = Haloperidol-*O*-TMS; 38 = Fluphenazine-*O*-TMS; 39 = Loratadine); (C) a real sample containing 113.6 ng/ml cocaine (12), 803.6 ng/ml benzoylecgonine (17), 74 ng/ml cocaethylene (14), 96.5 ng/ml ecgonine methylester (5), 262.3 ng/ml Δ-9-THC (19), 48.7 ng/ml cannabinol (23), 191.7 ng/ml cannabidiol (18), 5046.7 ng/ml MDMA (9), 2630.3 ng/ml MDA (7) and 50 ng/ml d_5-MDMA-*N*-TMS (8), d_3-Morphine-bis-*O*-TMS (25), d_3-Δ-9-THC-COOH-bis-*O*-TMS (32) as ISTDs.

were not amenable to a proper validation following international standards, as they showed to be quite dependent on the state of the chromatographic column. Nevertheless some of these compounds, like oxazepam, have been easily detected in cases of diazepam consumption in the framework of the MACIUS project. Discrepancies in the number of compounds listed between Table 1 (compounds assayed and detected) and Tables 2 and 3 (compounds that have been validated for their quantification) are explained for the above-reported reasons.

3.2. Validation results

Tables 2 and 3 summarize the method validation data. Linear calibration curves were obtained for the compounds of interest with a correlation coefficient (r^2) higher than 0.99 in all cases. The absolute analytical recoveries (mean ± S.D.) obtained after SPE extraction at different concentration levels showed that they were independent from the concentration tested (recoveries of the deuterated analogues used as ISTDs were the same of those from the non deuterated substances listed in Table 2). Limits of detection and quantification, intra-assay and inter-assay precision and accuracy were considered adequate for the purposes of the present study and coefficients of variation for precision and accuracy at LOQ were always better than 20%. With reference to the freeze/thaw stability assays for quality control samples, no relevant degradation was observed after any of the three freeze/thaw cycles, with differences from the initial concentration less that 10%.

3.3. Oral fluid samples

The method here presented is being applied to oral fluid samples collected from individuals injured by any mechanism,

Table 2
Method validation data

Analyte	LOD (ng/ml) ($n=4$)	LOQ (ng/ml) ($n=4$)	Recovery (mean ± S.D., $n=4$)[a]			Calibration slope (mean ± S.D., $n=3$)	Calibration intercept (mean ± S.D., $n=3$)	Determination coefficient (r^2)
			75 (ng/ml)	125 (ng/ml)	175 (ng/ml)			
Amphetamine	6.8	20.6	101.2 ± 15.8	68.7 ± 13.1	69.3 ± 15.0	0.0262 ± 0.0050	−0.0113 ± 0.0208	0.9907 ± 0.0001
Methamphetamine	6.9	20.9	52.6 ± 16.6	50.0 ± 14.7	48.6 ± 2.6	0.0088 ± 0.0018	0.1186 ± 0.0835	0.9908 ± 0.0010
MDMA	2.9	8.9	70.1 ± 16.7	82.0 ± 19.8	60.2 ± 4.5	0.0104 ± 0.0113	0.0252 ± 0.0369	0.9935 ± 0.0023
MDA	4.7	14.4	97.7 ± 16.5	69.2 ± 10.1	63.7 ± 17.6	0.0208 ± 0.0032	0.0477 ± 0.1095	0.9914 ± 0.0020
MDEA	5.0	15.0	68.9 ± 15.9	82.6 ± 3.2	47.6 ± 4.0	0.0164 ± 0.0171	−0.0480 ± 0.0724	0.9927 ± 0.0001
Phentermine	4.2	12.8	58.9 ± 13.9	54.7 ± 14.3	48.4 ± 5.1	0.0011 ± 0.0010	0.0056 ± 0.0013	0.9904 ± 0.0006
Δ-9-THC	0.6	1.9	82.3 ± 16.2	69.7 ± 9.2	51.7 ± 5.4	0.0189 ± 0.0052	−0,0195 ± 0.0188	0.9919 ± 0.0019
Δ-9-THC-COOH	1.6	4.8	87.9 ± 13.5	71.7 ± 10.2	66.2 ± 10.3	0.0238 ± 0.0008	0.0225 ± 0.0108	0.9976 ± 0.0011
Δ-9-THC-OH	4.2	12.7	83.9 ± 12.9	73.0 ± 0.2	50.1 ± 5.1	0.0528 ± 0.0077	−0.0257 ± 0.0606	0.9973 ± 0.0030
Cannabinol	1.9	5.6	68.2 ± 9.1	64.6 ± 9.6	62.3 ± 6.1	0.1055 ± 0.0015	−0.1554 ± 0.0268	0.9961 ± 0.0037
Cannabidiol	0.3	0.9	81.9 ± 10.6	66.0 ± 14.5	59.1 ± 5.5	0.0236 ± 0.0008	−0.0415 ± 0.0418	0.9954 ± 0.0033
Cocaine	1.4	4.1	77.4 ± 13.4	73.6 ± 17.1	84.3 ± 13.0	0.0318 ± 0.0007	−0.0009 ± 0.0238	0.9946 ± 0.0015
Benzoylecgonine	2.6	8.0	83.2 ± 12.0	63.9 ± 12.3	79.5 ± 5.9	0.0071 ± 0.0009	−0.0101 ± 0.0068	0.9943 ± 0.0039
Cocaethylene	2.4	7.2	73.3 ± 16.1	69.5 ± 19.1	89.1 ± 10.4	0.0327 ± 0.0017	−0.0111 ± 0.0116	0.9915 ± 0.0017
Ecgonine methylester	4.2	12.7	79.3 ± 7.3	58.3 ± 2.9	76.8 ± 17.7	0.0263 ± 0.0035	0.0263 ± 0.0035	0.9934 ± 0.0028
Morphine	2.2	6.5	72.9 ± 17.1	72.3 ± 16.9	71.9 ± 10.1	0.0315 ± 0.0048	0.0307 ± 0.0547	0.9938 ± 0.0018
6-MAM	0.9	2.9	78.5 ± 14.5	82.8 ± 8.1	67.2 ± 13.1	0.0227 ± 0.0020	−0.0207 ± 0.0300	0.9929 ± 0.0008
Codeine	2.,2	6.6	70.3 ± 10.0	70.9 ± 17.3	77.7 ± 10.5	0.0252 ± 0.0011	0.0043 ± 0.0387	0.9931 ± 0.0010
Diazepam	5.4	16.3	83.8 ± 11.4	67.8 ± 18.1	63.9 ± 5.7	0.0168 ± 0.0000	−0.7327 ± 0.0221	0.9947 ± 0.0002
Sertraline	6.2	18.6	63.9 ± 9.4	74.9 ± 23.3	46.5 ± 5.8	0.0165 ± 0.0185	0.0070 ± 0.1267	0.9964 ± 0.0019
Fluphenazine	6.3	19.1	79.5 ± 15.5	71.2 ± 3.2	80.2 ± 6.4	0.0821 ± 0.0037	0.5435 ± 0.3036	0.9926 ± 0.0017
Chlorpromazine	0.4	1.0	74.2 ± 13.0	79.5 ± 3.3	86.1 ± 7.5	0.1223 ± 0.0032	−0.4483 ± 0.0610	0.9954 ± 0.0021
Amitryptiline	3.4	10.2	89.1 ± 11.9	97.1 ± 2.3	94.0 ± 5.3	0.1664 ± 0.0041	−0.1688 ± 0.2073	0.9914 ± 0.0002
Clorazepate	5.1	15.5	93.9 ± 7.8	90.7 ± 9.8	93.9 ± 4.0	0.02892 ± 0.0031	−0,0911 ± 0.1873	0.9968 ± 0.0002
Haloperidol	2.4	7.2	87.4 ± 5.2	96.1 ± 5.7	93.5 ± 2.7	0.0647 ± 0.0043	−0.1834 ± 0.2752	0.9939 ± 0.0040
Flurazepam	3.6	10.9	90.0 ± 0.8	99.7 ± 3.0	92.2 ± 6.5	0.0836 ± 0.0085	−0.4407 ± 0.3380	0.9937 ± 0.0022
Diphenydramine	5.4	16.3	89.5 ± 3.6	96.1 ± 5.4	74.1 ± 11.7	0.0170 ± 0.0008	0.1580 ± 0.0424	0.9925 ± 0.0017
Gabapentine	6.2	18.9	19.7 ± 2.7	8.8 ± 1.1	8.2 ± 2.1	0.0053 ± 0.0011	0.0592 ± 0.0098	0.9903 ± 0.0014
Hydroxyzine	0.9	2.5	98.8 ± 0.4	79.1 ± 8.8	69.7 ± 8.3	0.0467 ± 0.0013	0.2751 ± 0.1489	0.9941 ± 0.0011
Loratadine	2.9	8.8	77.9 ± 12.4	72.4 ± 7.5	67.3 ± 5.7	0.0212 ± 0.0027	0.0726 ± 0.0784	0.9910 ± 0.0010

[a] S.D.: standard deviation.

Table 3
Intra ($n = 5$) and inter-assay ($n = 15$) precision and accuracy obtained from analytes under investigation

Analyte	Intra-assay						Inter-assay					
	Precision (R.S.D.%)			Accuracy (error%)			Precision (R.S.D.%)			Accuracy (error%)		
	Concentration (ng/ml)						Concentration (ng/ml)					
	30	75	125	50	75	125	30	75	125	30	75	125
Amphetamine	16.8	7.0	2.1	16.9	9.5	3.0	8.6	14.4	8.5	20.9	15.6	14.3
Methamphetamine	10.5	3.5	1.5	12.9	3.6	2.1	19.0	15.6	17.8	18.3	15.3	3.7
MDMA	8.5	4.7	4.7	11.1	5.7	2.6	15.3	6.2	18.9	14.2	19.2	13.2
MDA	2.6	4.5	12.5	9.8	3.2	5.3	2.7	5.6	7.7	23.5	20.5	17.5
MDEA	6.5	13.3	11.3	2.5	12.2	9.3	18.6	19.1	13.1	12.0	13.2	10.9
Phentermine	16.7	1.6	7.6	22.2	11.2	5.4	24.0	9.5	6.7	16.8	15.8	5.7
Δ-9-THC	4.1	2.1	9.1	11.0	12.1	6.5	8.5	12.0	9.1	17.1	12.2	15.6
Δ-9-THC-COOH	6.5	3.4	2.0	11.0	15.0	14.8	8.7	4.1	6.6	13.1	13.9	15.0
Δ-9-THC-OH	10.3	14.9	14.7	13.5	15.2	14.7	17.9	0.2	7.6	16.4	14.8	6.5
Cannabinol	13.2	11.4	4.8	19.7	12.6	10.1	14.2	10.5	1.2	17.3	12.2	8.9
Cannabidiol	6.0	14.1	5.3	19.4	9.1	14.8	9.3	14.1	5.7	18.3	14.6	13.1
Cocaine	3.0	7.3	10.0	15.0	4.9	11.6	9.1	9.2	9.2	16.0	4.4	6.4
Benzoylecgonine	2.5	12.8	5.1	2.0	11.3	6.9	3.7	8.6	4.1	2.9	13.9	11.2
Cocaethylene	6.6	9.9	15.5	12.2	7.0	10.7	9.1	12.0	15.5	10.8	12.6	10.7
Ecgonine methylester	4.5	7.8	1.7	5.0	7.3	1.5	15.3	9.3	6.9	13.1	13.2	13.4
Morphine	5.7	1.2	11.9	6.6	5.4	8.7	6.7	14.6	9.2	11.9	12.3	12.0
6-MAM	9.6	4.0	14.2	9.7	16.9	11.9	6.6	6.5	11.2	9.2	13.1	7.9
Codeine	6.3	14.3	5.3	5.0	11.9	7.3	9.1	10.6	10.8	8.9	12.1	9.0
Diazepam	1.4	4.9	10.8	8.2	9.8	10.7	11.3	4.9	9.4	8.8	9.8	7.0
Sertraline	1.4	4.9	10.8	8.2	9.8	10.7	11.3	4.9	9.4	8.8	9.8	7.0
Fluphenazine	16.8	15.7	5.6	20.8	10.3	16.5	12.4	8.7	5.6	23.8	9.9	18.1
Chlorpromazine	16.8	15.7	5.6	20.8	10.3	16.5	12.4	8.7	5.6	23.8	9.9	18.1
Amitryptiline	18.8	2.4	16.9	12.3	14.9	15.9	19.5	2.4	16.3	11.1	14.9	15.9
Clorazepate	5.5	13.9	0.5	10.4	9.8	4.9	13.2	13.7	6.8	10.7	10.7	5.4
Haloperidol	18.3	3.8	11.7	20.5	13.4	10.0	18.3	2.0	13.7	14.2	20.1	9.6
Flurazepam	18.3	3.8	11.7	20.5	13.4	10.0	18.3	2.0	13.7	14.2	20.1	9.6
Diphenydramine	7.8	15.1	12.7	6.5	9.7	14.6	14.2	14.9	12.7	12.6	14.5	16.5
Gabapentine	1.8	15.6	6.9	19.3	11.3	6.5	11.7	9.6	9.4	17.9	11.8	7.8
Hydroxyzine	21.9	0.2	9.6	17.8	8.1	6.1	26.5	19.8	6.7	24.9	14.6	6.3
Loratadine	0.2	10.7	25.6	10.4	8.1	17.0	20.5	10.3	14.8	17.7	7.1	13.7

such falls, road traffic, violence, sport, etc., which attended the emergency rooms of the above reported hospitals. Typical combinations of psychoactive substances and concentrations found are reported in Table 4 for selected oral fluid samples to show the applicability of the methodology described. While the MACIUS project has a larger scope than others recently developed like IMMORTAL (Impaired Motorists, Methods of Roadside Testing and Assessment for Licensing) more focused at roadside testing [28], both projects agree in the analytical approach to be applied in this type of studies.

Table 4
Analytes concentration (ng/ml) in oral fluid samples showing typical combinations of psychoactive drugs found in the MACIUS project

Compound (ng/ml)	A	B	C	D	E	F	G	H
MDMA				161.0				25233.7
MDA								13151.6
Δ-9-THC	220.6	73.5						1311.4
Cannabinol	43.2	30.6						243.5
Cannabidiol	131.1	46.4						958.3
Cocaine		65.5	2184.6					567.8
Benzoylecgonine		69.0	3273.7					4017.9
Cocaethylene			32.1					369.9
Ecgonine methylester			849.2					482.5
Morphine							34.6	
6-MAM							83.9	
Codeine					372.0			
Diazepam						1682.3		

4. Conclusions

The GC–MS method reported allows the determination of 30 psychoactive substances mainly related with drugs of abuse. The main characteristics of the assay are the rapid and simple extraction procedure and GC–MS analysis for the simultaneous measurement of several psychoactive compounds in oral fluid. Owing to the minimum handling, total time required and unequivocal detection of substances, this procedure can be routinely applied also in analytical laboratories of hospital emergency rooms.

The present analytical methodology, as discussed previously has been designed to evaluate the impact of the consumption of psychoactive substances on patients injured in any type of accident (MACIUS project). Preliminary results already indicate the adequacy of the experimental approach of performing the screening and quantification of psychoactive drugs in oral fluid and, using as analytical methodology GC/MS. While LC/MS is an alternative to GC/MS, the project would not be possible following an approach with an extensive use of immunoassays.

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