Determination of pharmaceutical and illicit drugs in oral fluid by ultra-high performance liquid chromatography - tandem mass spectrometry

Abstract

A simple and extremely fast procedure for the quantitative determination in oral fluid samples of 44 substances, including the most common drugs of abuse and several pharmaceutical drugs, was developed and fully validated. Preliminary sample treatment was limited to protein precipitation. The resulting acetonitrile solution was directly injected into an ultra-high performance liquid chromatograph (UHPLC) equipped with a C18 column (100 mm × 2.1 mm, 1.7 μm). The mobile phase eluted with linear gradient (water/formic acid 5 mM: acetonitrile/formic acid 5 mM; v:v) from 98:2 to 0:100 in 5.0 min, followed by isocratic elution at 100% B for 1.0 min. The flow rate was 0.6 mL/min and the total run time was 9.0 min including re-equilibration at the initial conditions. The analytes were revealed by a triple quadrupole mass spectrometer operating in the selected reaction monitoring mode. The method proved to be simple, accurate, rapid and highly sensitive, allowing the simultaneous detection of all compounds. The ease of sample treatment, together with the wide range of detectable substances, all with remarkable analytical sensitivity, make this procedure ideal for the screening of large populations in several forensic and clinical contexts, whenever oral fluid sampling has to be preferred to blood sampling, as for example in short retrospective investigations.

Highlights

► Oral fluid represents an important alternative to blood. ► Detection of pharmaceutical and illicit drugs in oral fluid is crucial. ► We developed a UHPLC–MS/MS method to detect 44 compounds in oral fluid. ► The method proved simple, accurate, rapid and highly sensitive.

Keywords

- Oral fluid;
- Ultra-high performance liquid chromatography;
- Multianalyte;
- Validation

1. Introduction

Over the past 10 years, oral fluid has progressively gained consideration as a valuable biological matrix for diagnostic purposes [1]. It is well known that oral fluid represents an important alternative to blood because it does not require invasive collection nor complex professional skills to be sampled. Further advantages of oral fluid analysis include minimal risk of contracting infections during sample collection, reduced risk of adulteration, and short detection window, which provides reliable indication of recent drug intake, unlike urine. Therefore, oral fluid analysis is likely to provide a cost-effective approach to the screening of large populations, and an useful tool in several forensic and clinical challenging situations, whenever blood sampling is difficult or impossible, such as in roadside testing, treatment facilities and prisons, and collection from children, handicapped, anxious or chronic pain patients [1], [2], [3], [4], [5] and [6]. Workplace drug testing programs also embraced oral fluid as a valuable testing matrix [7] and [8].

A major drawback of oral fluid sampling, is that an insufficient volume is frequently produced and collected. Therefore, the analytical methods have to be developed with the objective of using a minimal volume of oral fluid, especially when the collected fluid has to be used for both screening and confirmatory testing [9] or an aliquot has to be stored for subsequent investigation. The small sample volume available concurrently recalls the need of multianalyte methods. Confirmation tests need to cover a broad range of drugs and detect low analytes concentration, taking into account that the collection devices further dilute the collected oral fluid sample with a buffer solution, for stability purposes. Liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) allows the simultaneous detection of analytes of different polarity without derivatization, and assures excellent sensitivity. Several procedures on oral fluid have been proposed and largely listed and reviewed [10], [11] and [12]. Recently, ultra-high performance liquid chromatography (UHPLC) has been introduced to replace HPLC with the aim of obtaining faster analysis, less solvent consumption and improved resolution [13]. UHPLC is likely to be considered a particularly proper technique for the analysis of several analytes on small volumes of oral fluid [14], [15], [16], [17] and [18], for example the most common drugs of abuse and benzodiazepines, or the new designer drugs [19].

Aim of our study was to develop a fast and sensitive UHPLC–MS/MS method to detect several pharmaceutical and illicit drugs in oral fluid at once. All the most common drugs of abuse, i.e., the ones usually screened for with on-site immunoassay devices during roadside drug testing, were included, together with 17 benzodiazepines and metabolites, zolpidem, 9 opioids and metabolites for pain management and addiction control, and 9 antidepressants and neuroleptics, for a total of 44 substances. Some of these molecules are frequently involved in clinical contexts, such as the treatment of chronic pain, or in forensic investigations, with particular reference to the Italian territory. In comparison with the procedures previously reported, the present method used a simple sample treatment (protein precipitation) and direct injection into the UHPLC–MS system, avoiding

solid-phase or liquid–liquid extraction. Furthermore, the use of UHPLC–MS/MS technology allowed a drastic reduction of the analysis time without loss of resolution, and resulted in significantly reduced costs. The method proved to be simple, accurate, rapid and highly sensitive, allowing the simultaneous detection of most drugs of abuse and several pharmaceutical drugs, making this method ideal for clinical and forensic investigations on oral fluid.

2. Experimental

2.1. Chemicals and reagents

All reference and internal standards were purchased from either LGC Promochem SRL (Milan, Italy) or Sigma–Aldrich (Milan, Italy). Methanol, acetonitrile and sodium azide were provided by Sigma–Aldrich (Milan, Italy). Formic acid (LC–MS grade) was obtained by Fisher Scientific (Geel, Belgium). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). Stock standard solution were stored at −20 °C until used. Six deuterated compounds were used as the internal standards (IS): cocaine-d_3 (COC-d_3), amphetamine-d_6 (AMP-d_6), morphine-d_3 (MORP-d_3), Δ^9-tetrahydrocannabinol-d_3(THC-d_3), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine-d_3 (EDDP-d_3) and nitrazepam-d_5 (NIT-d_5). Two working solution mixtures were prepared by dilution in methanol at final concentrations of respectively 250 ng/mL (working solution A) and 1 µg/mL (working solution B). Lastly, an internal standard mixture working solution was prepared in methanol at the final concentrations of 10 µg/mL.

2.2. Sample preparation

Oral fluid samples were collected directly inside a tube containing about 10 mg of NaN_3 as a preservative, without stimulation. Each aliquot of neat oral fluid (500 µL) was fortified with 2 µL of internal standard mixture to yield a final concentration of 40 ng/mL. One milliliter of acetonitrile previously stored at −20 °C, was added to the sample, which was then incubated at −20 °C for 15 min. Afterwards, the sample was centrifuged at 4000 rpm for 15 min and a 50 µL of the organic phase was transferred into a new vial. Finally, the vial was centrifuged once more at 14,000 rpm for 10 min and a 4 µL aliquot was directly injected into the UHPLC–MS/MS system.

2.3. Instrumentation

Analyses were performed using a Shimadzu Nexera LC-30 A Series system (Shimadzu, Duisburg, Germany), interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray Turbo Ion source operating in positive-ion mode. A Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm), protected by a VanGuard C18 guard column (Waters Corporation, Italy), was used for the target analytes separation. The column oven was maintained at +50 °C and the elution solvents were water/formic

acid 5 mM (solvent A) and acetonitrile/formic acid 5 mM (solvent B). The mobile phase eluted under the following linear gradient conditions (A:B; v:v): from 98:2 to 0:100 in 5.0 min, followed by isocratic elution at 100% B for 1.0 min. The flow rate was 0.6 mL/min and the total run time was 9.0 min, including re-equilibration at the initial conditions. The triple-quadrupole mass analyzer operated in the selected reaction monitoring (SRM) mode. In order to establish appropriate SRM conditions, optimization of the mass spectrometer was conducted by direct infusion of the analytes into the electrospray ionization capillary and the declustering potential (DP) was adjusted to maximize the intensity of the protonated molecular species. For each SRM transition, the collision offset voltage values (CE) and the cell exit potentials (CXP) were also optimized. Each SRM transition was maintained during a time window of ±17.0 s around the expected retention time of the corresponding analyte, and the SRM target scan time (i.e., sum of dwell times for each SRM cycle) was 0.18 s, including pause times of 5 ms between consecutive SRM transitions. The best results were obtained using a source block temperature of +550 °C and an ion-spray voltage of +4000 V. Both Q1 and Q3 were operated at unit mass resolution.

Nitrogen was employed as the collision gas (5×10^{-3} Pa). The gas settings were as follows: curtain gas 30.0 psi, collision gas 8.0 psi, ion source gas (1) 45.0 psi, and ion source gas (2) 40.0 psi. The Analyst 1.5.2 (AB Sciex) software was used for data processing. All analytes and internal standards, their corresponding retention time, SRM transitions, and potentials are presented in Table 1.

Table 1.SRM transitions and experimental conditions for all compounds and internal standards detection.

	Compound	t_R(min)	Precursor ion	DP (V)	Target			Qualifier		
			$[M+H]^+$		Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
1	Alprazolam	2.9	309.0	77	280.9	36	12	205.1	56	9
								273.8	35	11
2	Amitriptyline	2.7	278.0	82	90.9	32	10	233.1	24	10
								104.9	31	10
3	Amphetamine	1.5	136.0	46	119.2	12	10	91.0	24	11
								65.0	46	9
4	Bromazepam	2.5	315.9	88	182.2	42	8	209.2	35	9
			318.0	88				182.0	43	16
5	Buprenorphine	2.4	468.3	40	55.1	95	10	414.3	47	10
								396.2	53	9
6	Carbamazepine	2.7	237.0	70	192.0	31	16	193.1	46	9
								194.1	26	17
7	Chlorpromazine	2.8	319.0	40	58.1	61	9	86.1	25	13
			320.9	40				58.0	63	8
8	Clonazepam	2.9	316.0	91	269.7	34	10	241.2	46	11
								214.1	51	17
9	Cocaine	2.0	304.1	75	182.0	26	12	82.1	37	8
								104.9	40	7
10	Codeine	1.4	300.0	38	165.2	38	59	152.2	80	9
								199.0	45	8
11	Delorazepam	3.2	304.9	27	139.9	39	21	242.1	37	11
								206.2	46	9
12	Desalkylflurazepam	3.1	289.0	79	139.9	38	12	226.0	38	16

	Compound	t_R(min)	Precursor ion	DP (V)	Target			Qualifier		
			$[M+H]^+$		Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
								104.1	68	17
13	Diazepam	3.4	285.0	51	154.0	36	7	193.1	43	15
								222.1	37	11
14	EDDP	2.5	278.2	85	186.2	45	11	234.2	35	11
								249.2	38	9
15	Fentanyl	2.3	337.0	175	132.2	42	12	105.1	50	10
								188.2	31	9
16	Flunitrazepam	3.1	314.0	35	268.2	36	11	239.1	47	11
								183.1	66	9
17	Fluoxetine	2.7	310.1	58	44.1	44	5	148.2	12	14
18	Flurazepam	2.4	388.1	33	314.9	33	14	316.9	26	12
			390.0	33				316.9	34	13
19	Ketamine	1.7	238.0	35	163.0	31	11	207.1	20	9
								125.0	38	11
20	Lorazepam	2.9	321.1	27	274.8	31	13	229.0	42	18
			323.0	27				277.0	34	12
21	MDA	1.5	180.0	44	133.1	25	12	135.1	25	8
								79.2	39	7
22	MDMA	1.5	194.1	48	105.1	34	8	163.2	18	11
								133.0	27	9
23	Methadone	2.7	310.0	80	265.1	20	10	105.0	33	9
								77.0	73	12
24	Methamphetamine	1.6	105.1	69	91.0	27	10	119.1	15	11
								65.0	54	8
25	Midazolam	2.4	325.9	55	291.0	36	9	208.9	46	7

	Compound	t_R(min)	Precursor ion	DP (V)	Target			Qualifier		
			$[M+H]^+$		Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
								223.0	50	9
26	Morphine	1.2	286.1	153	152.1	75	8	201.1	35	10
								165.0	56	7
27	Nitrazepam	2.8	282.1	30	236.0	32	11	180.0	50	9
								207.0	47	8
28	Norbuprenorphine	2.1	414.2	47	187.0	50	15	101.3	46	8
								339.9	42	17
29	Nordiazepam	3.0	271.0	71	140.0	37	13	208.0	38	18
								164.9	40	13
30	Norfentanyl	1.8	233.2	77	84.3	23	7	55.0	49	10
								56.2	40	8
31	Olanzapine	1.5	313.0	58	282.1	36	9	198.0	52	9
								256.1	33	11
32	Oxcarbamazepine	2.5	252.9	50	236.0	20	10	180.1	40	15
								208.0	28	9
33	Oxycodone	1.5	316.0	18	241.0	38	10	256.1	35	11
								298.1	27	12
34	Paroxetine	2.5	329.9	19	192.0	29	16	70.1	49	9
								150.9	32	20
35	Quetiapine	2.4	384.1	70	279.1	43	12	253.1	55	11
								221.1	67	9
36	THC	5.1	315.2	77	193.0	30	8	259.2	26	11
								123.2	43	9
37	Tramadol	1.9	264.1	35	58.1	46	11	246.1	15	14
38	Triazolam	3.0	343.0	36	308.0	37	13	314.9	39	13

	Compound	t_R(min)	Precursor ion	DP (V)	Target			Qualifier		
			$[M+H]^+$		Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
								238.9	54	10
39	Venlafaxine	2.2	278.1	55	260.2	17	11	58.0	22	10
								120.9	38	11
40	Zolpidem	2.1	308.1	50	235.1	47	21	263.1	34	12
								236.2	37	11
41	4-hydroxyalprazolam	2.8	325.1	75	216.1	51	11	204.9	59	12
								297.2	47	21
42	6-MAM	1.5	328.2	95	165.1	53	9	210.9	34	10
								193.2	37	9
43	7-aminoclonazepam	1.9	286.1	80	121.1	39	11	222.2	35	19
								195.2	47	9
44	7-aminonitrazepam	1.5	252.0	91	120.9	35	18	94.0	48	15
								224.2	30	10
IS	Morphine-d_3 (MORP-d_3)	1.2	289.1	153	152.1	75	8			
IS	Amphetamine-d_6 (AMP-d_6)	1.5	142.0	46	93.0	24	11			
IS	Cocaine-d_3 (COC-d_3)	2.0	307.1	75	185.0	26	12			
IS	EDDP-d_3	2.5	281.2	85	234.2	35	11			
IS	Nitrazepam-d_5 (NIT-d_5)	2.8	287.1	30	185.0	42	14			
IS	THC-d_3	5.1	318.2	77	196.2	30	8			

2.4. Method validation

The analytical method was validated in accordance with the recommendations of ISO/IEC 17025:2005 international standard. The following parameters were investigated: selectivity, linearity range, LOD and LOQ, intra-assay precision, accuracy, and recovery. Carry-over and matrix effect phenomena were also evaluated. Oral fluid was collected from ten healthy volunteers (five females, five males) and used as the working matrix for all validation experiments.

2.4.1. Identification criteria and selectivity

Identification criteria for the analytes were established according to national [20] and international guidelines[21] and [22]. Retention time is part of the acceptance criteria for chromatographic assays. In particular, deviations of 1–2% from the calibrators or controls are acceptable for LC based assays. When mass spectrometry is used for the identification of an analyte, the use of at least one qualifying mass transition for each analyte, in addition to the primary fragmentation, is recommended. Variations of mass transitions intensities were considered acceptable within ±20%, with respect to the corresponding control.

The repeatability of relative peak intensities for the SRM transitions of each analyte was determined on five spiked fresh oral fluid samples at two concentration levels (1 and 25 ng/mL for working solution A; 10 and 150 ng/mL for working solution B). Retention time (t_R) precision at each concentration was also determined. Furthermore, two pools of five fresh different blank oral fluid samples were analyzed as described above. For each analyte, the signal to noise ratio (S/N) was measured for the corresponding mass transitions at the expected retention time windows. A S/N < 3 was considered satisfactory in order to verify the method selectivity.

2.4.2. Linearity range and evaluation of LODs and LOQs

The linear calibration model was checked by analyzing (two replicates) blank oral fluid samples spiked with working solutions at six final concentrations. More in detail, the intervals 1–25 ng/mL (1, 2.5, 5, 10, 15 and 25 ng/mL) and 10–150 ng/mL (10, 25, 50, 75, 100 and 150 ng/mL) were investigated for the working solutions A and B, respectively. Quantitative data resulting from area counts were corrected using the respective IS signal areas. The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient, adjusted by taking into account the number of observations and independent variables (Adj R^2), was utilized to roughly estimate linearity. The appropriateness of the model was assessed by defining residuals and examining residual plots. The assumption of homoscedasticity was also successfully verified.

The limit of detection (LOD) was estimated as the analyte concentration whose response provided a S/N value equal to 3, as determined from the least abundant among qualifier SRM transitions; LOD was extrapolated from S/N values of the three lowest concentrations of the calibration curve.

The LOD values estimated from calculation were experimentally confirmed by analyzing spiked samples containing all analytes at concentrations approximately equal to their estimated LOD values. Similarly, the limit of quantification (LOQ) was estimated in the basis of the S/N ratio, which had to be equal or greater than 10 [23].

2.4.3. Precision and accuracy

For all analytes, intra-day and inter-day precision (expressed as percent variation coefficient, CV%) were evaluated by analyzing five oral fluid samples spiked at three concentration levels (1, 5 and 25 ng/mL for working solution A; 10, 50 and 150 ng/mL for working solution B) for three consecutive days. Accuracy (expressed as bias %) was assessed only within-run, since a new calibration curve is daily included in each analytical batch. Standard criteria for quantitative methods generally designated satisfactory assay precision when CV% values were below 15–20% for all concentration level while accuracy is considered satisfactory when the experimentally determined concentrations lied within ±15–20% from the expected values [23].

2.4.4. Matrix effect and extraction recovery

The matrix effect was calculated as the mean value obtained from five different oral fluid sources. Oral fluid samples were spiked after the extraction step at the final concentration of 1.0 and 10 ng/mL for working solution A and B respectively. For each analyte, the chromatographic peak area were compared with the mean peak area of three standard solutions prepared in acetonitrile, which is the solvent used for sample preparation and injection into the UHPLC system. Variability of matrix effect among different oral fluid sources was expressed as percent variation coefficient (CV%). The extraction recovery was calculated by comparing the experimental results of two sets of samples. In the first set, five oral fluid samples were spiked with all analytes at the final concentration of 25 and 150 ng/mL for working solution A and B respectively. In the second set, the spiking (at the same concentrations) was made on the blank oral fluid extracts [24].

2.4.5. Carry-over effect

The background chromatographic profiles for each analyte were monitored during the analysis of blank oral fluid sample injected for five times after the chromatographic run of a spiked blank oral fluid sample containing all the analytes at 25 ng/mL (working solution A) and 150 ng/mL (working solution B) concentration. To assure the absence of carry-over, the signal to noise ratio for each transition had to be lower than 3.

3. Results and discussion

3.1. UHPLC–MS/MS method

The optimized UHPLC–MS/MS method allowed the quantitative determination of 44 analytes and 6 internal standards. The whole chromatographic run, comprehensive of the time required for column re-equilibration before the following injection, was completed in 9.0 min. Retention times ranged between 1.2 min (morphine) and 5.1 min (THC). Fig. 1 shows the SRM chromatograms recorded from an oral fluid sample spiked with all analytes at 2.5 and 25 ng/mL concentration. These concentrations correspond to the second point of the calibration range for the substances contained in working solutions A and B, respectively (Table 2). Only one SRM transition is depicted for each analyte.

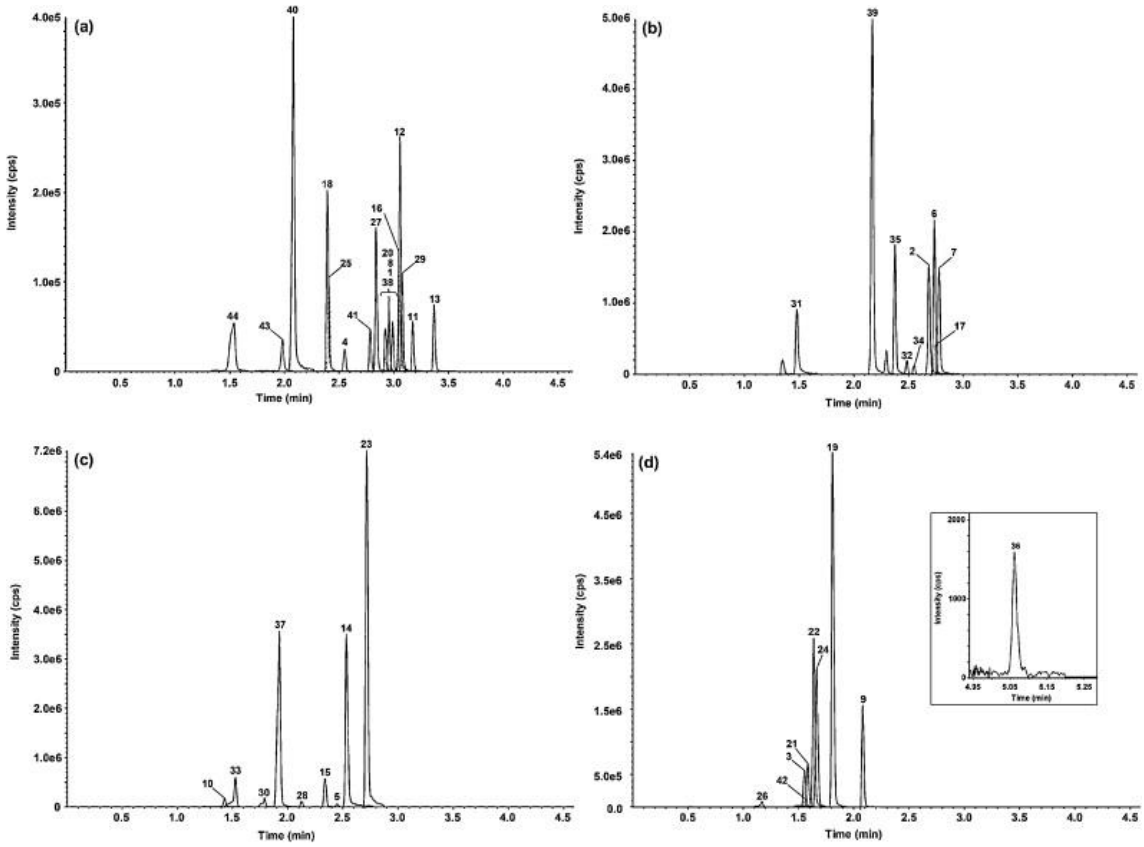

Fig. 1. ESI + SRM chromatograms for benzodiazepines and zolpidem (a), antidepressants and neuroleptics (b), opioids (c) and commons drugs of abuse (d). The chromatograms were recorded from an oral fluid sample spiked with the analytes at 2.5 ng/mL (working solution A) or 25 ng/mL (working solution B). Each analyte is labeled by the progressive number assigned as in Table 1. Only the target ion is shown.

Table 2. For each compound, the corresponding working solution and internal standard, linearity range, calibration curve, adjusted squared correlation coefficient, LOD and LOQ values are reported.

	Compound	Working solution	IS	Linearity range (ng/mL)	Calibration curve	Linearity (Adj R^2)	LOD (ng/mL)	LOQ (ng/mL)
1	Alprazolam	A	NIT-d_5	1–25	$y = 0.0931x + 0.0031$	0.9983	0.05	0.16
2	Amitriptyline	B	COC-d_3	10–150	$y = 0.009x + 0.0035$	0.9978	0.38	1.26
3	Amphetamine	B	AMP-d_6	10–150	$y = 0.085x + 0.1853$	0.9993	2.33	7.78
4	Bromazepam	A	NIT-d_5	1–25	$y = 0.0144x + 0.006$	0.9973	0.24	0.80
5	Buprenorphine	A	NIT-d_5	1–25	$y = 0.0007x + 9E-05$	0.9929	0.10	0.33
6	Carbamazepine	B	NIT-d_5	10–150	$y = 0.0286x + 0.1904$	0.9956	0.13	0.43
7	Chlorpromazine	B	MORP-d_3	10–150	$y = 0.0628x + 0.3125$	0.9869	0.40	1.34
8	Clonazepam	A	NIT-d_5	1–25	$y = 0.0294x - 0.002$	0.9913	0.21	0.71
9	Cocaine	A	COC-d_3	1–25	$y = 1.0399x + 1.2693$	0.9911	0.18	0.59
10	Codeine	B	MORP-d_3	10–150	$y = 0.0159x + 0.0537$	0.9888	1.04	3.48
11	Delorazepam	A	NIT-d_5	1–25	$y = 0.023x - 0.0029$	0.9989	0.11	0.36
12	Desalkylflurazepam	A	NIT-d_5	1–25	$y = 0.0417x - 0.0179$	0.9966	0.07	0.23
13	Diazepam	A	NIT-d_5	1–25	$y = 0.045x - 0.0059$	0.9981	0.02	0.07
14	EDDP	B	EDDP-d_3	10–150	$y = 0.0069x + 0.0222$	0.9993	0.96	3.19
15	Fentanyl	A	COC-d_3	1–25	$y = 0.0016x + 0.0006$	0.9901	0.11	0.38
16	Flunitrazepam	A	NIT-d_5	1–25	$y = 0.0674x - 0.027$	0.9925	0.10	0.32
17	Fluoxetine	B	COC-d_3	10–150	$y = 0.209x - 1.0234$	0.9950	0.37	1.24
18	Flurazepam	A	NIT-d_5	1–25	$y = 0.2076x + 0.2004$	0.9914	0.05	0.17
19	Ketamine	B	COC-d_3	10–150	$y = 0.023x - 0.0613$	0.9929	0.44	1.46
20	Lorazepam	A	NIT-d_5	1–25	$y = 0.0369x - 0.004$	0.9984	0.20	0.66
21	MDA	B	AMP-d_6	10–150	$y = 0.0335x + 0.0626$	0.9943	0.50	1.68
22	MDMA	B	AMP-d_6	10–150	$y = 0.1115x + 0.0482$	0.9941	0.42	1.38
23	Methadone	B	EDDP-d_3	10–150	$y = 0.0153x + 0.065$	0.9835	0.04	0.13

	Compound	Working solution	IS	Linearity range (ng/mL)	Calibration curve	Linearity (Adj R^2)	LOD (ng/mL)	LOQ (ng/mL)
24	Methamphetamine	B	AMP-d_6	10–150	$y = 0.0903x - 0.3231$	0.9966	0.37	1.22
25	Midazolam	A	NIT-d_5	1–25	$y = 0.0128x + 0.0069$	0.9990	0.06	0.22
26	Morphine	B	MORP-d_3	10–150	$y = 0.0228x + 0.0156$	0.9985	0.59	1.98
27	Nitrazepam	A	NIT-d_5	1–25	$y = 0.0586x - 0.0127$	0.9978	0.12	0.40
28	Norbuprenorphine	A	NIT-d_5	1–25	$y = 0.005x + 0.0035$	0.9970	0.26	0.85
29	Nordiazepam	A	NIT-d_5	1–25	$y = 0.08x - 0.0216$	0.9984	0.04	0.15
30	Norfentanyl	A	COC-d_3	1–25	$y = 0.2271x - 0.2278$	0.9955	0.29	0.96
31	Olanzapine	B	NIT-d_5	10–150	$y = 0.0206x + 0.0202$	0.9861	1.23	4.11
32	Oxcarbamazepine	B	NIT-d_5	10–150	$y = 0.0094x - 0.0291$	0.9903	0.48	1.60
33	Oxycodone	B	COC-d_3	10–150	$y = 0.0294x + 0.1577$	0.9906	0.68	2.26
34	Paroxetine	A	COC-d_3	1–25	$y = 0.0301x - 0.0182$	0.9844	0.30	0.99
35	Quetiapine	B	NIT-d_5	10–150	$y = 0.1868x + 1.0946$	0.9958	0.02	0.07
36	THC	A	THC-d_3	1–25	$y = 0.0242x - 0.0046$	0.9968	0.24	0.80
37	Tramadol	B	NIT-d_5	10–150	$y = 0.0086x - 0.0074$	0.9989	2.98	9.93
38	Triazolam	A	NIT-d_5	1–25	$y = 0.1346x - 0.0075$	0.9889	0.08	0.26
39	Venlafaxine	B	NIT-d_5	10–150	$y = 0.0894x + 0.4015$	0.9925	0.04	0.14
40	Zolpidem	A	NIT-d_5	1–25	$y = 0.3313x + 0.3892$	0.9929	0.06	0.19
41	4-hydroxyalprazolam	A	NIT-d_5	1–25	$y = 0.0254x + 0.0018$	0.9935	0.16	0.52
42	6-MAM	A	MORP-d3	1–25	$y = 0.1584x - 0.0239$	0.9921	0.21	0.70
43	7-aminoclonazepam	A	NIT-d5	1–25	$y = 0.0417x + 0.024$	0.9958	0.26	0.86
44	7-aminonitrazepam	A	NIT-d5	1–25	$y = 0.0723x + 0.058$	0.9988	0.29	0.98

3.2. Method validation

3.2.1. Identification criteria and selectivity

In order to achieve unambiguous identification, three SRM transitions were utilized for each analyte, as summarized in Table 1. Together with the retention time, these transitions provide more than necessary identification points to achieve unequivocal recognition of all analytes. The intra-assay precision for retention times, measured at low and high concentrations, showed random fluctuations within ±1.0%, confirming their repeatability, which is not affected by the analytes concentration. The temperature control of the UHPLC column oven, maintained at +50 °C, proved to be an important parameter to obtain repeatable retention times. For each analyte, the relative abundance of the three selected SRM transitions was found to vary by less than ±20%. Again, this variability meets the requirements for the unambiguous identification of all analytes included in the assay.

The SRM chromatograms obtained from two pools of blank oral fluid samples showed no interfering signals (i.e., S/N ratio minor than 3) at the expected retention time, for all analytes. This demonstrates that the method is selective for all tested compounds and free from positive interferences from oral fluid components and column bleeding.

3.2.2. Linearity and evaluation of LOD and LOQ

Table 2 reports the Adj R^2 values obtained from the calibration curves, that range from 0.9835 (methadone) up to 0.9993 (amphetamine and EDDP) and indicate good fit and linearity. All the back calculations of standards were within 15% at each calibration level. Table 2 also reports LOD and LOQ values, calculated from S/N values of the three lowest concentrations of the calibration curve. LOD values ranged from 0.02 ng/mL (diazepam and quetiapine) to 2.98 ng/mL (tramadol). LOQ values ranged correspondingly from 0.07 to 9.93 ng/mL. Positive detection (S/N > 3) of all analytes at their approximate LOD concentrations was confirmed experimentally. For most analytes, LOD values were lower than the 1 ng/mL limit without using large volumes of oral fluid (i.e., only 500 µL of oral fluid is consumed). Also LOQ values are significantly lower than the concentration levels expected for true positive samples, confirming that the present method is highly reliable and scarcely susceptible of yielding false-negative results. In this sense, the most relevant comparison can be made with the oral fluid cut-off values for illicit drugs, proposed by national [20] and international associations, such as European Workplace Drug Testing Society [25], for confirmatory analysis. In practice, the LOQ values are one order-of-magnitude smaller than the recommended cut-offs.

3.2.3. Precision and accuracy

Intra-assay data on precision and accuracy are reported in Table 3. The results demonstrated satisfactory intra-assay precision, as the percent variation coefficient (CV%) is lower than 15% (20% at the lowest concentration) for almost all analytes at three spiking concentrations: 1, 5 and 25 ng/mL for working solution A and 10, 50 and 150 ng/mL for working solutions B.

Table 3. For each compound, intra-assay precision (n = 5), trueness, recovery (high concentration) and matrix effect (low concentration) are reported.

	Compound	Low level					Medium level			High level			
		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	ME[c]		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	RE[d] (%)
					Mean (±%)	CV%							
1	Alprazolam	1	6.8	+20.2	−8.2	11.9	5	4.8	−5.2	25	5.8	−13.7	82
2	Amitriptyline	10	11.4	−2.4	−24.9	8.9	50	3.8	+0.4	150	7.9	+5.5	94
3	Amphetamine	10	5.8	+7.8	−9.8	10.2	50	3.4	+14.4	150	5.7	−7.6	89
4	Bromazepam	1	12.1	+15.5	−18.6	18.4	5	3.3	+12.6	25	6.5	−12.6	80
5	Buprenorphine	1	8.5	+1.3	−6.6	20.3	5	11.5	+0.8	25	11.5	+43.4	101
6	Carbamazepine	10	14.5	−3.0	−18.1	10.2	50	6.5	+6.5	150	5.1	+0.1	95
7	Chlorpromazine	10	9.3	+1.2	−17.4	14.0	50	12.8	+7.7	150	12.5	+3.7	109
8	Clonazepam	1	12.3	−12.7	−18.1	18.1	5	6.8	+7.0	25	4.3	−0.4	92
9	Cocaine	1	12.1	+7.0	−14.9	17.3	5	4.4	+15.8	25	6.8	−20.6	94
10	Codeine	10	3.4	+19.4	+0.9	11.2	50	10.0	+1.3	150	2.4	−10.9	116
11	Delorazepam	1	10.1	−3.1	−13.8	16.4	5	3.4	+4.7	25	6.6	−1.9	82
12	Desalkylflurazepam	1	7.8	−4.0	−13.7	14.1	5	5.2	−0.4	25	5.7	−2.9	82
13	Diazepam	1	8.1	−12.3	−11.4	14.5	5	6.2	+9.2	25	4.0	−1.3	80
14	EDDP	10	14.0	−1.2	+29.0	9.4	50	10.1	+9.1	150	8.0	−7.8	83
15	Fentanyl	1	10.7	+11.4	−16.3	24.7	5	14.8	+15.9	25	13.1	+11.9	92
16	Flunitrazepam	1	8.2	+14.0	−14.9	12.5	5	4.4	+9.1	25	10.4	−15.3	85
17	Fluoxetine	10	10.8	−11.6	−14.4	12.1	50	11.7	−1.4	150	11.8	−17.6	88
18	Flurazepam	1	13.3	+18.8	−15.9	21.0	5	10.9	+7.6	25	6.5	+14.9	85
19	Ketamine	10	15.4	−5.5	+21.9	13.4	50	7.5	+7.1	150	21.0	−11.8	98
20	Lorazepam	1	10.3	+14.2	−3.5	14.8	5	10.2	+7.4	25	4.6	−19.4	80

	Compound	Low level					Medium level			High level			
		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	ME[c]		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	RE[d] (%)
					Mean (±%)	CV%							
21	MDA	10	5.5	+9.2	+1.9	28.0	50	9.8	+12.4	150	8.3	+1.7	88
22	MDMA	10	6.4	−5.6	−26.1	19.2	50	8.3	+11.8	150	13.0	−9.2	87
23	Methadone	10	14.1	−1.3	−15.7	7.2	50	8.6	+4.2	150	4.0	−12.0	93
24	Methamphetamine	10	8.8	+1.7	−16.3	21.0	50	3.9	+1.1	150	10.0	−7.1	86
25	Midazolam	1	15.8	+2.8	−17.7	19.0	5	14.5	+19.4	25	11.1	−12.9	87
26	Morphine	10	6.3	+5.0	+7.7	7.8	50	3.4	+4.3	150	4.0	−5.7	90
27	Nitrazepam	1	11.3	+2.8	−9.5	13.9	5	3.2	+7.8	25	5.0	−11.6	80
28	Norbuprenorphine	1	15.9	+17.7	+2.3	21.7	5	6.6	+12.0	25	7.14	+1.2	101
29	Nordiazepam	1	8.5	+1.7	−8.6	15.4	5	5.3	+1.2	25	2.9	−4.4	82
30	Norfentanyl	1	5.1	+11.4	−0.8	24.5	5	7.2	−8.4	25	15.3	+0.5	77
31	Olanzapine	10	29.2	+13.4	−15.1	18.5	50	12.0	+7.8	150	15.6	−9.6	90
32	Oxcarbamazepine	10	8.0	−7.0	−30.1	16.4	50	12.2	+5.8	150	13.1	−15.7	93
33	Oxycodone	10	14.3	−13.3	+5.2	9.6	50	12.5	+2.0	150	12.6	+6.9	84
34	Paroxetine	1	11.8	−5.9	−15.7	25.4	5	14.2	+13.2	25	10.7	−19.7	79
35	Quetiapine	10	9.3	+19.8	−11.9	11.3	50	4.0	+12.1	150	9.7	−11.6	89
36	THC	1	3.3	+13.6	−10.8	14.1	5	4.2	+5.7	25	4.8	−14.1	84
37	Tramadol	10	8.7	+21.0	−20.4	14.0	50	5.3	−1.4	150	6.0	−19.5	89
38	Triazolam	1	7.5	+9.9	−4.7	20.9	5	7.1	−0.2	25	6.4	−19.3	76
39	Venlafaxine	10	13.0	−17.1	−20.6	14.3	50	5.0	+8.2	150	6.8	−19.7	90
40	Zolpidem	1	13.0	+8.7	−9.9	17.3	5	11.3	+21.8	25	15.7	−12.6	87
41	4-hydroxyalprazolam	1	16.7	+4.0	+6.1	20.7	5	3.8	+1.3	25	1.7	−7.9	84
42	6-MAM	1	16.3	−5.4	+3.7	25.0	5	8.2	+10.6	25	4.8	+21.7	84

	Compound	Low level					Medium level			High level			
		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	ME[c]		Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	Conc. (ng/mL)	PR[a] (CV%)	AC[b] (bias%)	RE[d] (%)
					Mean (±%)	CV%							
43	7-aminoclonazepam	1	6.0	+11.0	−1.2	9.9	5	14.7	−0.4	25	13.6	−20.6	80
44	7-aminonitrazepam	1	10.4	+21.0	+9.9	14.5	5	9.3	+21.4	25	3.2	+21.5	86

[a]Intra-assay precision (n = 5).

[b]Accuracy.

[c]Matrix effect.

[d]Recovery.

At the lower concentration, all tested analytes showed CV values below 17% except olanzapine (29.2% at 10 ng/mL), while at medium concentration all the responses lied within the satisfactory limits. At high concentration, ketamine showed the largest but still acceptable CV value (21.0%) (Table 4).

Table 4. For each compound, inter-assay precision (n = 15) at low, medium and high concentration are reported.

	Compound	Low level		Medium level		High level	
		Conc. (ng/mL)	PR[a] (CV%)	Conc. (ng/mL)	PR[a] (CV%)	Conc. (ng/mL)	PR[a] (CV%)
1	Alprazolam	1	19.5	5	14.2	25	9.1
2	Amitriptyline	10	9.8	50	6.5	150	4.1
3	Amphetamine	10	11.9	50	4.7	150	4.7
4	Bromazepam	1	18.7	5	10.6	25	9.4
5	Buprenorphine	1	19.9	5	17.3	25	16.5
6	Carbamazepine	10	9.2	50	5.3	150	6.3
7	Chlorpromazine	10	14.5	50	13.1	150	4.5
8	Clonazepam	1	14.6	5	8.4	25	5.6
9	Cocaine	1	17.6	5	5.1	25	6.9
10	Codeine	10	17.8	50	19.2	150	12.3
11	Delorazepam	1	13.4	5	5.5	25	5.7
12	Desalkylflurazepam	1	13.9	5	6.4	25	4.8
13	Diazepam	1	13.2	5	7.0	25	4.4
14	EDDP	10	10.2	50	8.1	150	4.5
15	Fentanyl	1	14.7	5	17.7	25	10.9
16	Flunitrazepam	1	16.8	5	7.4	25	11.7
17	Fluoxetine	10	8.1	50	10.0	150	13.9
18	Flurazepam	1	19.2	5	15.6	25	10.9
19	Ketamine	10	11.9	50	9.3	150	13.7
20	Lorazepam	1	14.3	5	10.4	25	6.7
21	MDA	10	13.5	50	8.2	150	6.9
22	MDMA	10	10.9	50	9.2	150	7.0
23	Methadone	10	8.1	50	7.6	150	7.7
24	Methamphetamine	10	4.4	50	3.5	150	6.2
25	Midazolam	1	17.7	5	14.5	25	7.2
26	Morphine	10	6.1	50	3.8	150	7.0
27	Nitrazepam	1	13.8	5	6.4	25	4.6

	Compound	Low level		Medium level		High level	
		Conc. (ng/mL)	PR[a] (CV%)	Conc. (ng/mL)	PR[a] (CV%)	Conc. (ng/mL)	PR[a] (CV%)
28	Norbuprenorphine	1	12.7	5	13.7	25	8.1
29	Nordiazepam	1	15.3	5	6.3	25	5.0
30	Norfentanyl	1	14.3	5	13.3	25	6.6
31	Olanzapine	10	19.5	50	15.6	150	14.9
32	Oxcarbamazepine	10	13.2	50	12.6	150	10.8
33	Oxycodone	10	12.7	50	11.6	150	10.5
34	Paroxetine	1	19.9	5	17.7	25	14.2
35	Quetiapine	10	9.0	50	7.7	150	11.9
36	THC	1	14.3	5	6.8	25	5.2
37	Tramadol	10	10.3	50	10.1	150	12.6
38	Triazolam	1	19.5	5	19.1	25	13.3
39	Venlafaxine	10	10.3	50	8.6	150	11.9
40	Zolpidem	1	18.7	5	14.5	25	6.9
41	4-hydroxyalprazolam	1	18.4	5	19.7	25	11.0
42	6-MAM	1	15.3	5	10.6	25	17.1
43	7-aminoclonazepam	1	17.1	5	15.6	25	6.0
44	7-aminonitrazepam	1	12.2	5	8.7	25	14.9

[a]Inter-assay precision (n = 15).

The CV% for inter-day precision never exceeded the limit of 20%. The accuracy, expressed as percent bias, was satisfactory for all compounds except for buprenorphine that was overestimated at 25 ng/mL (+43.4%). In general, the calculated biases were in the interval ±22% at both low and high concentrations. In particular, at 1 ng/mL, bias values ranged from −12.7% (clonazepam) to +21.0% (tramadol and 7-aminonitrazepam), while at 10 ng/mL, bias values ranged from −17.1% (venlafaxine) to +19.8% (quetiapine). At 5 ng/mL, bias values ranged from −8.4% (norfentanyl) to +21.8% (zolpidem), while at 50 ng/mL bias were between −1.4% (fluoxetine and tramadol) and +14.4% (amphetamine). At the higher concentration level, bias values ranged from −20.6% (cocaine and 7-aminoclonazepam) to +21.7% (6-MAM) at 25 ng/mL and from −19.7% (venlafaxine) to +6.9% (oxycodone) at 150 ng/mL.

3.2.4. Matrix effect and extraction recovery

For each analyte, the matrix effect was evaluated at the low concentration range, while the extraction recovery was determined at higher concentration. The results are shown in Table 3.
The variability among five different oral fluid samples was acceptable (CV% < 25%) except for MDA (28%), so we decided to pool together the sources of oral fluid to perform the validation experiments for linearity, evaluation of LODs and LOQs, precision, accuracy, recovery and carry-over. For almost all analytes, the matrix effect proved to be negative, i.e., signal suppression is observed. The highest negative effect was seen for oxcarbamazepine at 10 ng/mL (−30.1%), while the largest positive value was +29.0% for EDDP at 10 ng/mL. Ion suppression is quite common in ESI, whenever a complex mixtures is studied, since co-elution of analytes and extraneous substances makes the competition for the charge dependent on their relative chemical and physical properties. In the present case, the modest oral fluid sample clean-up and co-elution of some analytes, due to the short chromatographic run, are most likely to produce the observed matrix effect. In particular, protein precipitation does not completely remove the endogenous substances, such as lipids and phospholipids, that may play some role in the ESI droplet desolvation process. However, signal suppression does not affect significantly the detection capability of this method, since LOD values for all analytes are still lower than the expected concentrations in real oral fluid samples, and co-elution of analytes is generally not presumed to occur in real samples. These minor drawbacks are largely counterbalanced by the global analytical workflow, which is maintained simple and fast, as is nowadays requested in clinical and forensic laboratories in order to increase the laboratory throughput and decrease analysis-time and costs.
The extraction efficiency for the proposed method proved satisfactory, with recovery values ranging from 76% for triazolam to 116% for codeine.

3.2.5. Carry-over effect

No carry-over effects were observed under the conditions described in the experimental section. Blank oral fluid samples, alternatively analyzed with samples spiked at high concentration (25 and 150 ng/mL), showed S/N values always lower than 3 at the retention times of the tested analytes.

3.2.6. Application to real cases

Our laboratory is continuously using the present method for the routine analysis of real samples, mainly from roadside testing, drug abuse withdrawal control and therapeutic monitoring. The experimental activity concerning oral fluid is controlled by periodic external Proficiency Tests, namely “Drugs in Oral Fluid Scheme” provided by LGC Standards Proficiency Testing. Two examples of multiple positive identifications are reported, in order to demonstrate the practical

usefulness and general applicability of this method. In Fig. 2, the chromatograms of samples respectively positive to THC (left), and paroxetine and alprazolam (right) are reported.

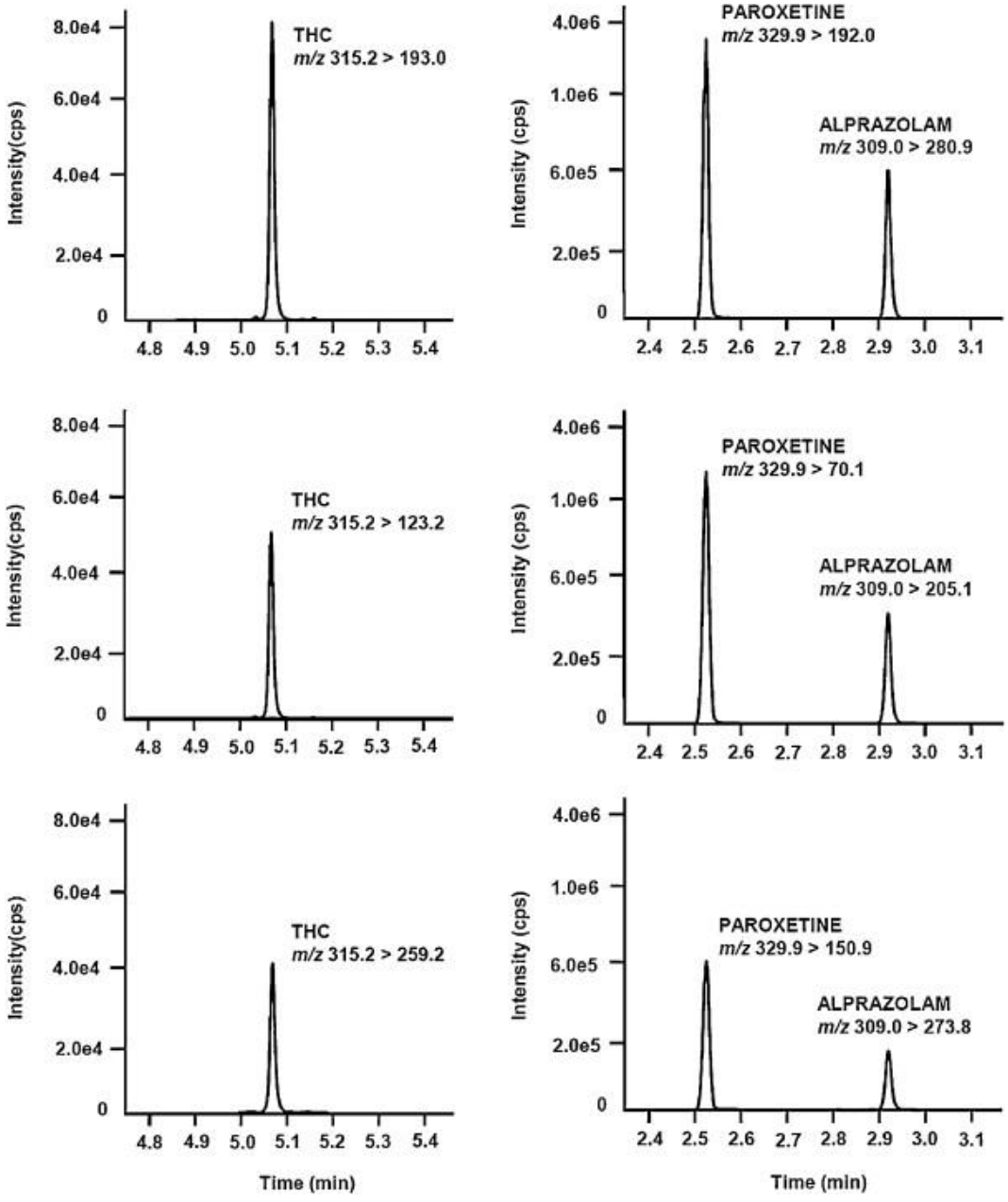

Fig. 2. (Left) ESI + SRM chromatograms of a real sample positive to THC (concentration: 24 ng/mL) and (right) ESI + SRM chromatograms of a real sample positive to positive to paroxetine (concentration: 108 ng/mL) and alprazolam (concentration: 10 ng/mL).

4. Conclusions

A simple and fast procedure was developed and fully validated for the simultaneous quantification in oral fluid samples of 44 substances of clinical and forensic interest. In comparison with previously published papers, main features of the proposed method are: easier and faster sample processing; wider range of analytes; higher analytical sensitivity. The use of UHPLC–MS/MS instrumentation provided an efficient combination of chromatographic resolution, high speed, sensitivity and selectivity, that is exploited to reduce the gap between screening and confirmatory methods, as it combines specificity and accuracy requirements with high efficiency and high throughput objectives.

The present protocol positively satisfies different key-features of most clinical and forensic investigations, especially when large populations have to be screened for a high number of substances. These are (i) accurate and precise quantification of common drugs of abuse and pharmaceutical compounds in oral fluid samples, as an alternative to blood, whose sampling often encounters practical limitations, (ii) fast processing and reporting, as is increasingly requested to improve laboratory throughput, and by police forces and/or physicians, who are frequently called to adopt fast and reliable legal and/or clinical actions, and (iii) simultaneous detection of a wide range of substances with variable physical–chemical properties. Lastly, the adoption of an unselective preliminary sample treatment together with the inherent flexibility of the UHPLC–MS/MS protocol allow easy expansion of the analytical method to encompass more drugs, either new or becoming important in clinical and forensic investigations.

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