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LC–MS/MS method for the determination of nine antidepressants and some of their main metabolites in oral fluid and plasma Study of correlation between venlafaxine concentrations in both matrices

A. de Castro, M. Concheiro, O. Quintela, A. Cruz, M. López-Rivadulla*

Forensic Toxicology Service, Institute of Legal Medicine of the University of Santiago de Compostela, C/San Francisco s/n, 15782 Santiago de Compostela, Spain

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ABSTRACT

In this paper, a fast, sensitive and selective LC–MS/MS method is described for the simultaneous determination of amitriptyline, imipramine, clomipramine, fluoxetine, paroxetine, sertraline, fluvoxamine, citalopram and venlafaxine, as well as some of their main metabolites (nortriptyline, desipramine, norclomipramine and norfluoxetine), in oral fluid and plasma. The sample (0.2 mL) was extracted with an automated solid-phase extraction system (ASPEC XL), using mixed mode OASIS MCX cartridges. Chromatographic separation was performed in a Sunfire C18 IS column (20 mm × 2.1 mm, 3.5 μm), using a gradient of acetonitrile and ammonium formate (pH 3; 2 mM) as mobile phase, which allowed the elution of all the compounds in less than 5 min. The method has been fully validated in both specimens. This method was initially applied to the analysis of oral fluid and plasma samples from patients on antidepressant treatment in order to assess for which compounds it was likely to find a good correlation between both matrices. The best results were obtained for venlafaxine, so the study was extended for this compound, comparing the ratio between oral fluid and plasma concentrations ($R_{OF/PL}$) in five patients on venlafaxine treatment when both samples were collected simultaneously on four different occasions. An important inter and intraindividual variability was found in oral fluid concentrations for 150 mg dose (mean = 287.5 ng/m, range 58.8–531.2 ng/mL) and for 75 mg dose (mean = 186.3 ng/mL, range = 82.1–289.2 ng/mL). $R_{OF/PL}$ was calculated for each patient on the four different occasions, showing also a high variability (CV = 24.2–69.6%).

1. Introduction

Antidepressants are drugs widely used in different psychiatric disorders. Within the general denomination of antidepressants, a wide range of compounds with very heterogeneous structures are included. For this reason, these compounds are usually classified taking into account its mechanism of action [1].

Although tricyclic antidepressants (TCAs) continue to be used in special situations, such as refractory and severe depression, new antidepressants now substitute old ones in most applications. For this reason, fatal cases associated with antidepressant overdoses are less frequent than in the past [2,3]. Generally, overdoses with the new antidepressants are less frequently fatal and, in the cases where death occurred, usually other substances were also detected, ascribing the cause of death to intoxication by the action of multiple drugs [4–7].

With regard to therapeutic drug monitoring (TDM) of antidepressants, this practice is supported by several authors for TCAs because of two main reasons: their narrow therapeutic window with high risk of cardiotoxicity and CNS toxicity [8–14], and the high intra and interindividual variability in the concentrations reached at a given dose [2–8]. In the case of the new generations of antidepressants, TDM is not justified routinely because of their wide therapeutic window and the relative safety of these compounds compared to the high toxicity of TCAs. Nevertheless, it could be useful in special situations, such as elderly, slow or rapid metabolizers, polimedicated patients and when changing the treatment or suspecting patient non-compliance [8,9,11,14].

Plasma is the main biological sample used for TDM purposes, as it represents the concentration of the analyte responsible for the pharmacological effect, as well as the side and toxic effects. However, oral fluid as an alternative to plasma samples has been studied for TDM of different compounds [15–19]. This specimen shows several advantages: painless and non-invasive collection which does not require qualified personnel, it can be easily obtained on several occasions, and it represents the free analyte fraction. However, this

* Corresponding author. Tel.: +34 981582327; fax: +34 981580336.
E-mail address: apimlriv@usc.es (M. López-Rivadulla).

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specimen has also some disadvantages, like the small volume of sample usually available and the fact that several factors can affect the diffusion of the analytes from plasma to oral fluid (pH, oral contamination, collection by stimulation vs. non-stimulation). For these reasons, correlation between plasma and oral fluid concentrations should be studied before using this alternative specimen for TDM purposes.

Several methods have been developed for the determination of antidepressants by LC–MS, most of them for the determination of one compound and its main metabolites [20–24], or some compounds belonging to the same antidepressant group [25–29].

Shinozuka et al. published a method for the determination in plasma of 20 different antidepressants with a chromatographic run time of 30 min [30]. Also Kirchherr et al. developed a method for the TDM of 48 antidepressants and antipsychotics, using different volumes of the reconstitution solvent for different groups of antidepressants [31]. Recently, Sauvage et al. [32], as well as de Castro et al. [33] have developed on-line LC–MS/MS methods for the determination of the main marketed antidepressants in serum and plasma, respectively.

In relation to oral fluid, Pujadas et al. [34] and Wiley et al. [35] have reported GC-MS methods for the determination of different psychoactive drugs, including sertraline and amitriptyline in the first case, and the main antidepressants in the second.

The aim of this study was the development of an LC–MS/MS method for the determination of the main marketed antidepressants in oral fluid and plasma samples. The method was also applied to the analysis of oral fluid samples from patients under different antidepressant treatment to study the possibility of using this alternative specimen for TDM purposes.

2. Materials and methods

2.1. Chemicals and reagents

All individual standards and deuterated internal standards (IS) were purchased from Cerilliant (Round Rock, TX, USA), except nortriptyline, norclomipramine and venlafaxine, which were obtained in solid form from Fluka–Sigma–Aldrich Chemie (Steinheim, Switzerland). LC–MS Lichrosolv acetonitrile (99.98% pure) was from Riedel de Häen–Sigma–Aldrich Chemie (Schnelldorf, Germany). Methanol, dichloromethane, 2-propanol, formic acid (98–100%), acetic acid (100%), sodium acetate and ammonium in solution 25% were from Merck (Darmstadt, Germany). Ammonium formate was from Fluka–Sigma–Aldrich Chemie (Steinheim, Switzerland). Purified water was obtained in the laboratory using a Milli-Q water system (Le Mont-sur-Lausanne, Switzerland). Oasis MCX cartridges 3 cm^3 60 mg were from Waters Corporation (Mildford, USA) and Microcon filter devices Ultracel YM-3 from Millipore Corp. (Bedford, MA, USA).

2.2. Specimens for method development

Blank oral fluid samples were collected from healthy volunteers by direct spitting in polypropylene tubes. Blank plasma samples were supplied by a local blood centre.

2.3. Preparation of calibration standards

Stock solutions of each standard at 1 mg/mL were diluted in methanol to prepare individual working solutions at 0.1 mg/mL, which were stored at −20 °C in the dark for a maximum of 6 months. A mixed working solution of all the compounds at a concentration of 2 μg/mL was daily elaborated in water to prepare the appropriate solutions for the calibration curve. A mixed working solution of the internal standards (IS) (nortriptyline-d_3, imipramine-d_3, clomipramine-d_3, paroxetine-d_6, norfluoxetine-d_6 and fluoxetine-d_6) at the appropriate concentration for each sample was also prepared by dilution in methanol.

2.4. Extraction procedure

An automated solid-phase extraction (SPE) system ASPEC XL (Gilson, Middletown, USA) and mixed mode OASIS MCX cartridges 3 cm^3 60 mg (Waters Corporation) were employed. Before the extraction, the ASPEC XL was used to add 1 mL of sodium acetate buffer pH 3.6 and 50 μL of the IS mixture (at 0.2 mg/L in oral fluid and 0.4 mg/L in plasma) to 0.2 mL of sample.

After conditioning the SPE cartridges with 2 mL methanol and 2 mL water, the samples were applied. Clean-up was accomplished with successive 2 mL washes of formic acid 2% in water and methanol. Cartridges were then dried by positive pressure with a flow of nitrogen for 5 min before elution with 2 mL of dicloromethane/2-propanol/ammonium hydroxide (75:24.5:0.5). The elution solution was evaporated to dryness at 35 °C under a stream of nitrogen. The dried extract was re-dissolved in 200 μL of a mixture of ammonium formate buffer (pH 3.0; 2 mM)-acetonitrile (85:15, v/v) for plasma samples, and 100 μL for oral fluid samples. The sample was subsequently transferred to autosampler vials, and 20 μL were injected onto the LC–MS.

2.5. Liquid chromatography/tandem mass spectrometry

The HPLC system was a Waters Alliance 2795 Separation Module with a Waters Alliance series column heater/cooler (Waters). A Sunfire C18 (20 mm × 2.1 mm, 3.5 μm) Intelligent Speed™ column was employed for the chromatographic separation of the antidepressants, using ammonium formate buffer (pH 3.0; 2 mM) and acetonitrile as mobile phase, at a flow rate of 0.4 mL/min. The column temperature was kept at 26 °C. The following gradient was applied: 15% acetonitrile until minute 0.5; then, acetonitrile percentage was gradually increased to 50% until minute 4, to increase again to 70% at minute 5. With these conditions, all the compounds eluted within 5 min, with a total run time of 8 min.

For the detection, a tandem mass spectrometer Quattro Micro™ API ESCI (Waters) with a triple quadrupole was employed. The instrument was operated in electrospray in the positive ionization mode (ESI+). Nitrogen was used as nebulization and desolvation gas at a flow rate of 800 L/h, heated to 400 °C, and as cone gas at a flow rate of 50 L/h. Capillary voltage and source block temperature were 0.5 kV and 130 °C, respectively.

The optimal cone voltage value to obtain the most prominent pseudomolecular ion $[M+H]^+$ for each compound, as well as the multiple reaction monitoring (MRM) transitions for the detection of each compound, were selected by infusion of individual solutions of each antidepressant into the mass spectrometer (10 μg/mL in mobile phase at a flow rate of 10 μL/min) in "T" with the effluent of the chromatographic system (ACN:ammonium formate 2 mM pH 3, 50:50, at a flow rate of 0.25 mL/min). Collision induced dissociation (CID) was performed using argon as collision gas at a pressure of 3×10^{-6} bar, and optimal collision energy values to obtain the most abundant fragments were established. Data acquisition was controlled using MassLynx 4.0 software and processed with QuanLynx 4.0 software (Waters). In Table 1, the MRM method that was employed is shown.

2.6. Validation study

Validation of the analytical methods in oral fluid and plasma was performed following the recommendations of different publications on that subject [36–43].

Table 1
MRM method for the studied compounds, as well as their retention times and the deuterated analogue used as internal standard for each compound

Compound	CV	Transition	CE	Dwell time (ms)	t_R	IS
Venlafaxine	25	278.1 > 57.8	18	0.15	0.77	Imipramine-d_3
		278.1 > 260.3	12	0.15		
Citalopram	35	325.1 > 109.1	26	0.15	2.63	Imipramine-d_3
		325.1 > 262.1	20	0.15		
Desipramine	25	267 > 43.8	40	0.05	3.4	Nortriptyline-d_3
		267 > 71.8	15	0.05		
Imipramine	25	281.1 > 57.6	40	0.05	3.51	Imipramine-d_3
		281.1 > 85.9	16	0.05		
Paroxetine	35	330 > 69.7	30	0.05	3.61	Paroxetine-d_6
		330 > 192.3	20	0.05		
Nortriptyline	25	264.2 > 90.8	22	0.05	3.72	Nortriptiline-d_3
		264.2 > 233.3	16	0.05		
Fluvoxamine	25	319 > 70.8	18	0.05	3.8	Paroxetine-d_6
		319 > 86.8	18	0.05		
Amitriptyline	30	278.1 > 90.8	24	0.05	3.82	Imipramine-d_3
		278.1 > 233.2	18	0.05		
Imipramine-d_3	25	284.1 > 89	15	0.05	3.51	
Paroxetine-d_6	35	336.1 > 76	30	0.05	3.59	
Nortriptyline-d_3	30	267.1 > 233.3	15	0.05	3.72	
Norfluoxetine	15	296 > 29.8	10	0.05	4.31	Norfluoxetine-d_6
		296 > 134.1	6	0.05		
Sertraline	18	306 > 159.1	26	0.05	4.41	Fluoxetine-d_6
		306 > 275.1	12	0.05		
Fluoxetine	22	310.1 > 43.7	12	0.05	4.45	Fluoxetine-d_6
		310.1 > 148.2	8	0.05		
Norclomipramine	25	301.1 > 71.8	18	0.05	4.46	Fluoxetine-d_6
		301.1 > 270.2	16	0.05		
Clomipramine	22	315 > 57.8	32	0.05	4.63	Clomipramine-d_3
		315 > 85.9	20	0.05		
Norfluoxetine-d_6	15	302.2 > 140.1	6	0.05	4.27	
Fluoxetine-d_6	25	316.1 > 43.7	12	0.05	4.41	
Clomipramine-d_3	30	318.1 > 88.9	18	0.05	4.63	

Underlined transitions are the ones used as quantifiers. CV: Cone voltage (V); CE: collision energy (eV); t_R: retention time (min); IS: internal standard.

Selectivity was evaluated by the analysis of blank oral fluid and plasma samples from 10 different people who were not on antidepressant treatment [36]. Besides, real plasma samples sent to our Toxicology Service containing other substances like benzodiazepines and/or drugs of abuse were also analyzed [37].

To study the best calibration model adjusted to our data, calibration curves were analyzed on six different days. Calibration curves were generated with eight concentration levels in the range from 2 to 500 ng/mL for oral fluid samples, and at nine concentration levels from 2 to 1000 ng/mL in the case of plasma samples [38,39].

The lower limit of quantification (LLOQ) was defined as the lower concentration that could be quantified with coefficient of variation (CV) and mean relative error (MRE) < 20% [38].

Within-day precision and accuracy were studied at three concentration levels (low, medium and high concentration) by analysis of five replicates for each concentration level on the same day. Between-day precision and accuracy were studied by analysis of the same concentration levels on five different days [38].

Recovery was evaluated at two concentration levels (low and high). For each compound and at each concentration level, the signal obtained when the analytes were added to a blank sample before extraction ($n = 5$) were compared to the signal obtained when the same amount of analytes were added after extraction ($n = 5$) [40]. In both cases, the same volume of the internal standard mixture was added after the extraction.

Matrix effect was evaluated initially by the post-column infusion experiment [41]. Post-column infusion of a mixture containing the studied compounds and the internal standards (1 μg/mL, 10 μL/min) was performed in "T" with the effluent of the chromatographic system. With this configuration, the chromatograms after the injection of blank oral fluid and plasma samples extracted as described before ($n = 6$ for each specimen) were compared with the chromatograms after the injection of mobile phase (no matrix effect). A second experiment consisted of comparing the signal after the analysis of blank oral fluid and plasma extracts spiked with a mixture of the standards ($n = 6$ for each specimen) with the signal after the injection of the standards dissolved in mobile phase [42].

Besides retention time and the selected MRM transitions for each compound, the relative ions intensity could be used as an additional confirmation parameter. According to a paper previously published by our team [43], the ion ratio was calculated as the peak area of the quantitation transition/the peak area of the qualifier transition. The relative ion intensity was defined as the percentage that the peak area of the qualifier transition supposed with regard to the quantifier transition (100/ion ratio). Within-day precision in this parameter was calculated at four concentration levels by the analysis of five replicates on the same day. Between-day precision was calculated at the same concentration levels analyzed on five different days.

Table 2
Calibration parameters and LLOQ in oral fluid (OF) and plasma (PL) for each compound

Compound	Matrix	LLOQ (ng/mL)	$y = a + bx$ or $y = a + bx^2 + cx$			
			Slope b	Slope c	Intercept (a)	r^2
Venlafaxine	OF	2	0.0155 ± 0.0007		0.00361 ± 0.00385	0.9972 ± 0.00171
	PL	2	0.00617 ± 0.0017		0.00112 ± 0.00253	0.9981 ± 0.0016
Citalopram	OF	2	0.01591 ± 0.0015		−0.00297 ± 0.00675	0.9973 ± 0.0015
	PL	2	0.00582 ± 0.0002		−0.00127 ± 0.00174	0.9970 ± 0.0022
Desipramine	OF	2	0.0741 ± 0.0050		0.01019 ± 0.04141	0.9971 ± 0.00149
	PL	2	0.03142 ± 0.0029		0.02317 ± 0.01465	0.9974 ± 0.0016
Imipramine	OF	2	0.02496 ± 0.0013		−0.00518 ± 0.01336	0.9971 ± 0.00198
	PL	2	0.0097 ± 0.0004		−0.00135 ± 0.00396	0.9978 ± 0.0019
Paroxetine	OF	2	0.03229 ± 0.0015		−0.01642 ± 0.02765	0.9968 ± 0.00183
	PL	2	0.00123 ± 0.0005		−0.00512 ± 0.00337	0.9983 ± 0.0013
Nortriptyline	OF	2	0.03405 ± 0.0018		−0.00844 ± 0.02536	0.9973 ± 0.0015
	PL	2	0.00129 ± 0.0008		−0.00305 ± 0.00477	0.9981 ± 0.00131
Fluvoxamine	OF	2	0.02428 ± 0.0016	$-9.99-\times-10^{-6} \pm 3-\times-10^{-6}$	0.00833 ± 0.02520	0.9968 ± 0.00227
	PL	10	0.00914 ± 0.0010	$-2.33 \times 10^{-6} \pm 7 \times 10^{-7}$	0.03814 ± 0.01836	0.9969 ± 0.00132
Amitriptyline	OF	2	0.01027 ± 0.0006		0.00270 ± 0.00736	0.9960 ± 0.00234
	PL	4	0.00403 ± 0.0002		0.00365 ± 0.0022	0.9980 ± 0.00092
Norfluoxetine	OF	2	0.02281 ± 0.0014		−0.00774 ± 0.02313	0.9962 ± 0.00200
	PL	4	0.00863 ± 0.0003		0.00142 ± 0.00845	0.9978 ± 0.00107
Sertraline	OF	2	0.04149 ± 0.0066		−0.02183 ± 0.03957	0.9957 ± 0.00164
	PL	2	0.01525 ± 0.0026		−0.00466 ± 0.000668	0.9978 ± 0.00180
Fluoxetine	OF	2	0.03184 ± 0.0021		−0.01989 ± 0.02891	0.9966 ± 0.00193
	PL	2	0.01236 ± 0.0009		−0.00389 ± 0.00627	0.9982 ± 0.00124
Norclomipramine	OF	2	0.04591 ± 0.0169		−0.00181 ± 0.03951	0.9965 ± 0.00200
	PL	2	0.02001 ± 0.0038		0.00401 ± 0.00686	0.9975 ± 0.00149
Clomipramine	OF	10	0.02082 ± 0.0007		−0.01252 ± 0.01802	0.9968 ± 0.00217
	PL	10	0.00779 ± 0.0005		−0.00399 ± 0.00262	0.9983 ± 0.00146

Stability of the antidepressants after three freeze/thaw cycles was evaluated in triplicate at two concentration levels (low and high). The signal for each compound in the samples subjected to the freeze/thaw cycles (stability samples) were compared to those obtained in freshly prepared samples (control samples) [40]. Stability and control samples were quantified with a calibration curve prepared on the day that the analysis was performed.

2.7. Oral fluid–plasma correlation preliminary study

Real oral fluid and plasma samples from patients under antidepressant treatment were taken to investigate the correlation of concentrations between both biological samples.

All patients involved in this investigation were previously informed about the characteristics of the study and they were asked to sign an informed consent to participate. Relevant information about the age, sex, time of sample collection and of dosing, antidepressant posology and concomitant treatment was also taken.

At first, a preliminary study was performed to assess which of the studied antidepressants was likely to show a good correlation between plasma and oral fluid levels. For this purpose, plasma and oral fluid samples were collected simultaneously twice from patients on different antidepressants treatment. The best results were obtained for venlafaxine, so the study was extended for this antidepressant.

In the venlafaxine study, oral fluid and plasma samples were collected simultaneously on four different occasions from five patients. The average age of the patients was 46.4 years old, and four out of five were women. In most of the cases, patients were taking concomitant medication. In all the cases, venlafaxine was administered in retard formulations. Within the five patients, three of them were taking daily doses of 150 mg, and the other two were taking 75 mg. Samples were collected, when possible, before the next dose, to ensure the drug was in the elimination phase rather than in the absorption or distribution ones, as there is more variability in these two pharmacokinetic processes after oral administration [44]. The dose and the time of sample collection was the same in the four different occasions for each individual patient. Oral fluid samples were collected by direct spitting into polypropylene tubes. Plasma samples were collected in heparinized tubes.

Both types of specimens were stored at −20 °C until their analysis. For the analysis, oral fluid and plasma samples were centrifuged at 14×10^3 rpm for 10 min, and 0.2 mL of the supernatant were extracted.

In addition, plasma samples were also filtered to study the correlation between the concentrations in the plasmatic free fraction and in oral fluid. For these purpose, 0.5 mL of plasma samples were filtered using Microcon filter devices Ultracel YM-3 (Millipore Corp.), centrifugated for 15 min at 14×10^3 rpm, and 0.2 mL of the filtered sample were extracted as described previously and analyzed.

3. Results and discussion

3.1. LC–MS/MS method

The described methodology allows the detection of the main marketed antidepressant and some of their metabolites in oral fluid and plasma, using the same sample extraction procedure for both specimens. For sample extraction, MCX mixed mode cartridges

Table 3
Results for within-day and between-day precision and accuracy for all the antidepressants in oral fluid

Compound	Concentration (ng/mL)	Within-day precision and accuracy ($n=5$)		Between-day precision and accuracy ($n=5$)	
		CV	MRE	CV	MRE
Venlafaxine	4	3.9	5.3	6.6	6.9
	100	6.8	3.6	10.0	1.6
	500	6.3	9.3	2.3	0.7
Citalopram	4	2.5	1.0	7.4	3.7
	100	7.9	−0.6	6.5	3.9
	500	5.1	7.6	2.5	2.3
Desipramine	4	7.7	−1.1	9.6	0.4
	100	7.9	−0.6	6.5	3.9
	500	7.4	12.9	2.5	−0.2
Imipramine	4	3.9	−2.0	7.8	1.5
	100	6.5	−2.6	8.0	2.0
	500	4.5	7.1	2.5	1.2
Paroxetine	4	6.0	−2.2	6.8	−0.0
	100	−3.9	7.0	1.2	−3.9
	500	4.1	10.7	3.2	1.6
Nortriptyline	4	9.1	−4.2	8.3	−0.1
	100	9.0	−2.5	6.1	2.4
	500	6.4	11.0	2.1	0.6
Fluvoxamine	4	7.3	−7.8	16.8	−7.9
	100	5.6	−1.8	7.25	−0.3
	500	10.6	8.7	1.4	0.6
Amitriptyline	4	9.4	−6.7	8.0	−5.8
	100	5.1	2.5	8.4	6.8
	500	4.5	4.1	3.4	−0.4
Norfluoxetine	4	9.7	−6.5	12.7	−12.2
	100	5.4	0.5	7.4	5.1
	500	4.7	11.3	2.6	−0.1
Sertraline	4	13.8	−10.7	10.4	−7.7
	100	7.3	−1.8	7.6	4.2
	500	8.9	3.0	1.7	1.9
Fluoxetine	4	4.8	−3.7	14.9	−5.4
	100	6.5	0.0	8.4	2.0
	500	4.3	10.5	2.3	1.5
Norclomipramine	4	6.2	−6.1	9.5	−8.4
	100	8.4	1.0	8.4	5.8
	500	3.7	7.4	2.8	0.5
Clomipramine	4	8.6	0.8	8.2	−2.6
	100	7.6	−4.8	7.2	0.2
	500	4.7	6.7	2.4	1.8

were selected as they retain the compounds by both, reverse-phase and cation exchange mechanism, so they allow obtaining cleaner extracts for basic compounds.

Some carry-over after the extraction using the SPE robot was found, even checking different cleaning steps after the extraction. However, the area was 30 times lower than the area at the LLOQ (2 ng/mL) for most of the compounds, so it did not affect the results reliability. Only for clomipramine, carry over was half of the signal of the LLOQ. For this reason, LLOQ was established for this compound at 10 ng/mL, both in oral fluid and plasma, even though the accepted criteria with regard to linearity, precision and accuracy were satisfied at a concentration of 2 ng/mL.

For the chromatographic separation of the compounds, a Sunfire C18 Intelligent Speed (IS) column was employed. These short analytical columns allow an important reduction in the total run time as higher flow rates can be employed keeping column pressure under 3000 psi, without decrease in resolution. This way, under the selected chromatographic conditions, all the compounds eluted within 5 min. Figs. 1 and 2 show the chromatograms of the 13 antidepressants at the LLOQ for most of the compounds (2 ng/mL) in oral fluid and plasma samples, respectively.

The selectivity of the method was verified as no interferences were found at the retention time of any of the compounds in their MRM channels when blank samples or real cases positive to drugs of abuse and other medicines like benzodiazepines were analyzed.

The linearity of the compound-to-IS peak ratio versus the theoretical concentration was verified in both matrices by using a $1/x$ weighted linear regression for all the compounds, except for fluvoxamine, for which a quadratic response was observed. The use of quadratic models is recommended if the accepted criteria are not satisfied with the linear model; however, more concentration levels are needed to define the calibration range [37,38]. Coefficient of determination (r^2) was >0.99 for all the compounds from 2 to 500 ng/mL in oral fluid, and from 2, 4 or 10 to 1000 ng/mL in plasma. The calibration range was higher for plasma as the method in this specimen would also be applied to judicial samples, were concentrations above the therapeutic ones could be found.

Table 4
Results for within-day and between-day precision and accuracy for all the antidepressants in plasma

Compound	Concentration (ng/mL)	Within-day precision and accuracy ($n = 5$)		Between-day precision and accuracy ($n = 5$)	
		CV	MRE	CV	MRE
Venlafaxine	20	1.8	4.5	3.7	−1.2
	500	4.8	−11.2	4.7	−2.2
	1000	6.8	6.1	2.4	2.3
Citalopram	20	1.5	3.9	5.5	−3.6
	500	5.5	−10.2	5.5	−10.2
	1000	5,6	11.4	2.2	3.4
Desipramine	20	0.8	10.9	6.0	2.9
	500	4.4	−8.0	2.1	0.3
	1000	3.1	4.8	1.9	−0.3
Imipramine	20	1.3	−2.2	5.0	−3.2
	500	6.5	−11.2	2.9	−0.9
	1000	5.3	8.3	2.4	2.0
Paroxetine	20	0.9	−6.9	5.0	−7.1
	500	5.7	−10.0	2.6	2.1
	1000	5.0	5.9	1.3	0.5
Nortriptyline	20	1.7	0.0	5.8	−5.0
	500	6.1	−7.9	2.9	1.1
	1000	4.2	7.2	1.8	0.8
Fluvoxamine	20	6.6	−4.9	8.0	−1.6
	500	1.5	−15.6	3.5	−0.9
	1000	5.4	−4.0	1.3	0.9
Amitriptyline	20	1.5	3.9	6.2	3.1
	500	5.8	−8.5	2.4	−0.2
	1000	5.6	8.3	1.9	−0.8
Norfluoxetine	20	1.8	2.3	6.2	−0.4
	500	7.6	−4.6	2.8	0.2
	1000	4.5	7.6	3.3	−2.7
Sertraline	20	1.4	−4.3	10.3	−3.9
	500	7.1	−5.9	3.0	0.1
	1000	8.0	13.9	2.4	0.6
Fluoxetine	20	2.9	−2.2	5.	−3.5
	500	7.3	−7.5	1.6	0.7
	1000	3.8	3.0	2.0	0.4
Norclomipramine	20	2.1	−3.3	6.1	−1.4
	500	5.6	−7.7	3.5	1.0
	1000	8.0	13.9	2.5	0.1
Clomipramine	20	1.2	−5.2	4.9	−6.6
	500	6.9	−7.6	2.8	1.2
	1000	7.5	−0.9	9.4	16.8

The LLOQ was 2 ng/mL in oral fluid, and 2, 4 or 10 ng/mL in plasma, depending on the compound. Table 2 shows the LLOQ, as well as the calibration parameters for each compound in oral fluid and plasma.

Within-day and between-day precision and accuracy were satisfactory for all the tested concentrations [38] (Tables 3 and 4).

The calculated recoveries were included between 49% and 72%. Recovery was a little low, but these values are accepted provided that the quantitation of the compounds is precise and accurate [37].

Values of CV for the relative ions intensities were included between 0.23% and 17% for most of the compounds, with a higher variability for norfluoxetine (CV < 26%).

No significant matrix effect was observed when performing the post-column infusion experiment. Fig. 3 shows the TIC (total ion current) after the injection of mobile phase (A) and an extracted blank oral fluid sample (B) simultaneous to the infusion of the antidepressants. A decrease in the signal after the injection of the oral fluid extract is observed at the beginning of the chromatogram, but before the elution of the first compound. In the second experiment, matrix effect was calculated quantitatively. Matrix effect was found to be <15%, except for norfluoxetine in both matrices (enhancement of the signal between 38% and 45%) and paroxetine in oral fluid (enhancement of the signal around 30%). However, this matrix effect could be compensated by using the deuterated IS for these compounds.

Stability studies of the analytes after three freeze/thaw cycles of plasma and oral fluid samples indicate that all the compounds are stable when subjected to these conditions (CV and MRE $\leq$20%), except in the case of sertraline in oral fluid, for which a slight decrease in the signal at 250 ng/mL was found (CV = 6.0% and MRE = −33.4%).

3.2. Oral fluid–plasma correlation preliminary study results

Table 5 shows the preliminary study results, in which different antidepressants were evaluated by collecting two oral fluid and plasma samples from each patient. As can be seen, the best results

Table 5
Oral fluid/plasma correlation study

Patient	Administered AD	Week	C_{PL} (ng/mL)	C_{OF} (ng/mL)	$R_{OF/PL}$	CV $R_{OF/PL}$
1	Venlafaxine 150 mg retard 1 dose/day	1	Venlafaxine = 179.8	Venlafaxine = 234.5	1.30	2.7
		2	Venlafaxine = 152.8	Venlafaxine = 206. 9	1.35	
2	Sertraline 100 mg, 1 dose/day	1	Sertraline = 43.1	Sertraline = 4.2	0.097	56.2
		2	Sertraline = 40.5	Sertraline = 1.8	0.044	
3	Clomipramine 75 mg, 1 dose/day	1	Clomipramine = 42.4	Clomipramine = 4.4 (<LLOQ)	0.104	103.7
		2	Clomipramine = 51.1	Clomipramine = 0.8 (<LLOQ)	0.016	
		1	Norclomipramine = 130.6	Norclomipramine = 14.8	0.113	42.0
		2	Norclomipramine = 110.0	Norclomipramine = 2.6 (<LLOQ)	0.024	
4	Venlafaxine 150 mg retard 1 dose/day	1	Venlafaxine = 62.6	Venlafaxine = 346.3	5.532	18.4
		2	Venlafaxine = 186.2	Venlafaxine = 792.8	4.258	
5	Citalopram 20 mg retard 1 dose/day	1	Citalopram = 49.3	Citalopram = 16.5	0.335	5.7
		2	Citalopram = 46.6	Citalopram = 14.4	0.309	
6	Venlafaxine 150 mg retard 2 dose/day	1	Venlafaxine = 256.2	Venlafaxine = 544.6	2.126	2.3
		2	Venlafaxine = 257.9	Venlafaxine = 566.1	2.195	
7	Paroxetina 20 mg, 1 dose/day	1	Paroxetine = 51.8	Paroxetine = 5.6	0.108	93.5
		2	Paroxetine = 57.8	Paroxetine = 1.3 (<LLOQ)	0.022	
8	Citalopram 30 mg, 1 dose/day	1	Citalopram = 34.7	Citalopram = 7.6	0.219	36.6
		2	Citalopram = 91.6	Citalopram = 34.1	0.372	
9	Venlafaxina 150 mg retard 1 dose/day	1	Venlafaxine = 51.4	Venlafaxine = 51.5	1.001	26.8
		2	Venlafaxine = 93.2	Venlafaxine = 137.0	1.470	
10	Sertralina 100 mg, 1 dose/day	1	Sertraline = 13.1	Sertraline = 0.7 (<LLOQ)	0.053	18.0
		2	Sertraline = 27.1	Sertraline = 1.1 (<LLOQ)	0.041	
11	Paroxetine 20 mg, 1 dose/day	1	Paroxetine = 9.1	Paroxetine = 17.1	1.879	49.7
		2	Paroxetine = 38.6	Paroxetine = 34.8	0.901	
12	Paroxetine 20 mg, 1 dose/day	1	Paroxetine = 3.0	Paroxetine = 1.8	0.600	17.2
		2	Paroxetine = 10.0	Paroxetine = 4.7	0.470	
13	Fluoxetine 20 mg, 1 dose/day	1	Fluoxetine = 11.7	Fluoxetine = 48.2	4.120	112.5
		2	Fluoxetine = 46.5	Fluoxetine = 21.8	0.469	
		1	Norfluoxetine = 9.5	Norfluoxetine = 9.2	0.968	84.5
		2	Norfluoxetine = 39.8	Norfluoxetine = 9.7	0.244	
14	Amitriptylina 50 mg, 1 dose/day	1	Amitriptyline = 3.2	Amitriptyline = 28.4	8.875	140.1
		2	Amitriptyline = 18.0	Amitriptyline = 33.1	1.839	
		1	Nortriptyline = 2	Nortriptyline = 21.3	10.650	90.3
		2	Nortriptyline = 8.6	Nortriptyline = 20.2	2.349	
15	Paroxetine 20 mg, 1 dose/day	1	Paroxetine = 2.1	Paroxetine = 2	0.952	15.5
		2	Paroxetine = 1.6	Paroxetine = 1.9	1.187	
16	Escitalopram 20 mg, 1 dose/day	1	Citalopram = 96.3	Citalopram = 113.3	1.176	14.8
		2	Citalopram = 52.1	Citalopram = 75.6	1.451	
17	Fluoxetine 20 mg, 1 dose/day	1	Fluoxetine = 100.6	Fluoxetine = 36.8	0.366	33.7
		2	Fluoxetine = 99.3	Fluoxetine = 22.3	0.225	
		1	Norfluoxetine = 95.6	Norfluoxetine = 26.3	0.275	44.2
		2	Norfluoxetine = 113.2	Norfluoxetine = 16.0	0.141	
18	Escitalopram 15 mg, 1 dose/day	1	Citalopram = 33.3	Citalopram = 76.4	2.294	10.8
		2	Citalopram = 34.3	Citalopram = 67.5	1.968	
19	Escitalopram 20 mg, 1 dose/12 h	1	Citalopram = 71.6	Citalopram = 63.2	0.883	43.3
		2	Citalopram = 67.6	Citalopram = 55.1	0.815	
20	Venlafaxine 150 mg retard 1 dose/day	1	Venlafaxine = 123.7	Venlafaxine = 593.1	4.795	13.6
		2	Venlafaxine = 150.0	Venlafaxine = 592.6	3.951	
21	Citalopram 20 mg, amitriptyline 10 mg, 1 dose/day	1	Citalopram = 52.5	Citalopram = 170.5	3.245	40.8
		2	Citalopram = 64.5	Citalopram = 379.1	5.877	
		1	Amitriptyline = 1.5 (<LLOQ)	Amitriptyline = 10.1	6.733	111.3
		2	Amitriptyline = 0.2 (<LLOQ)	Amitriptyline = 11.3	56.500	
		1	Nortriptyline = 2	Nortriptyline = 4.2	2.100	69.6
		2	Nortriptyline = 1.7	Nortriptyline = 10.5	6.176	

Preliminary results.

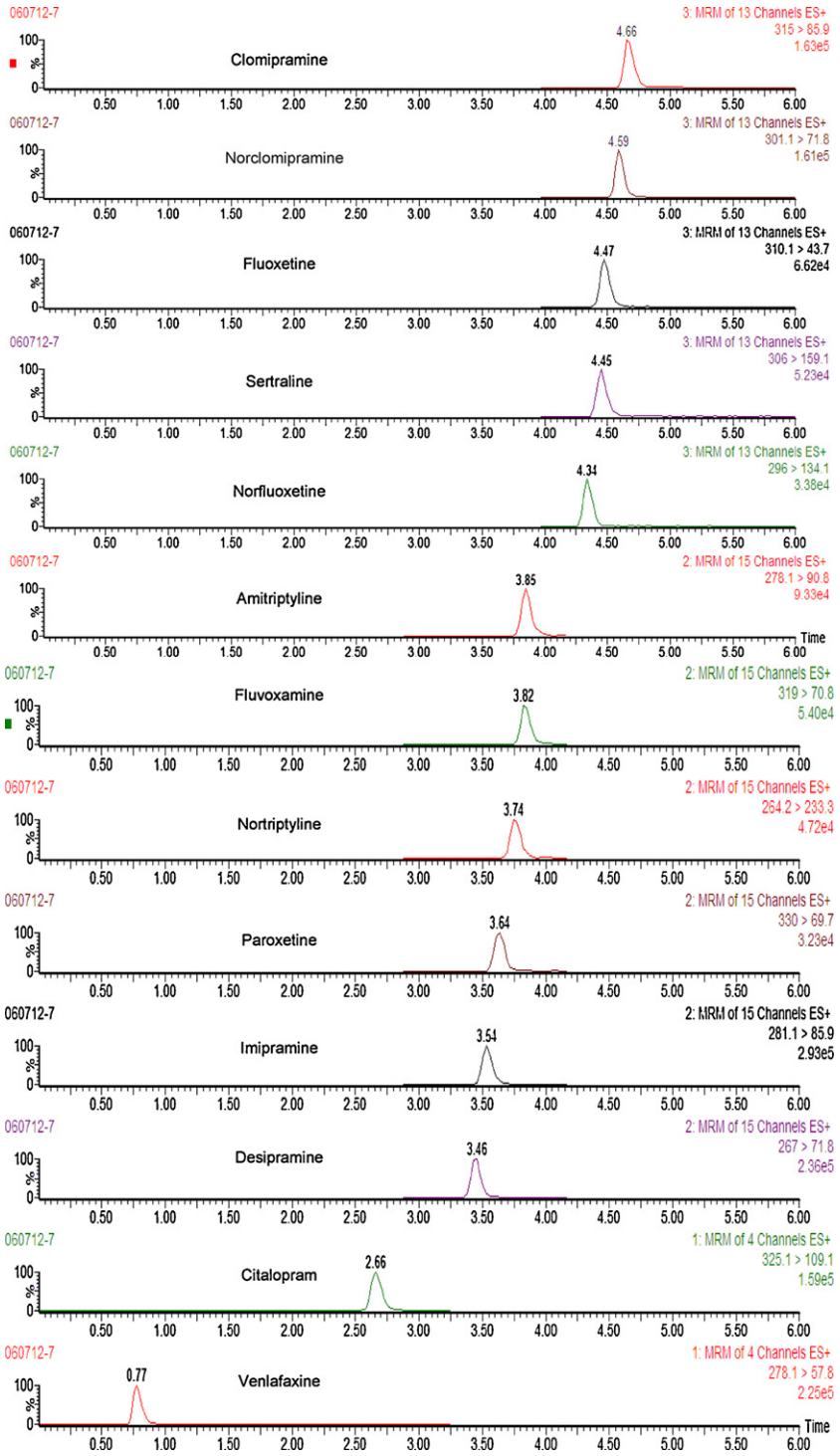

Fig. 1. Chromatograms of the quantifier transitions selected for each antidepressant in oral fluid at the LLOQ (2 ng/mL).

were obtained for venlafaxine, for which the coefficient of variation (CV) in the ratio between the concentrations in plasma and oral fluid ($R_{OF/PL}$) was <27% in all cases. For this reason, this study was extended for venlafaxine.

In the venlafaxine study, $R_{PL/OF}$ was calculated for each individual patient under venlafaxine treatment. Besides, as mainly the plasmatic free protein fraction is the one that reaches the plasma–oral fluid equilibrium [45], plasma was filtered to eliminate the proteins, and the ratio between the concentrations in the plasmatic free fraction vs. the concentration in oral fluid ($R_{OF/PL\text{-}FF}$) was also calculated.

The results were not analyzed interindividually as only five patients were included in the study and they were taking different daily doses.

Intraindividual results are shown in Table 6. For each patient, plasmatic concentrations (C_{PL}) were similar in the four analyzed samples (CV = 11–24%), but higher differences in oral fluid concentrations (C_{OF}) were found (CV = 16–63%). Also, a high variability

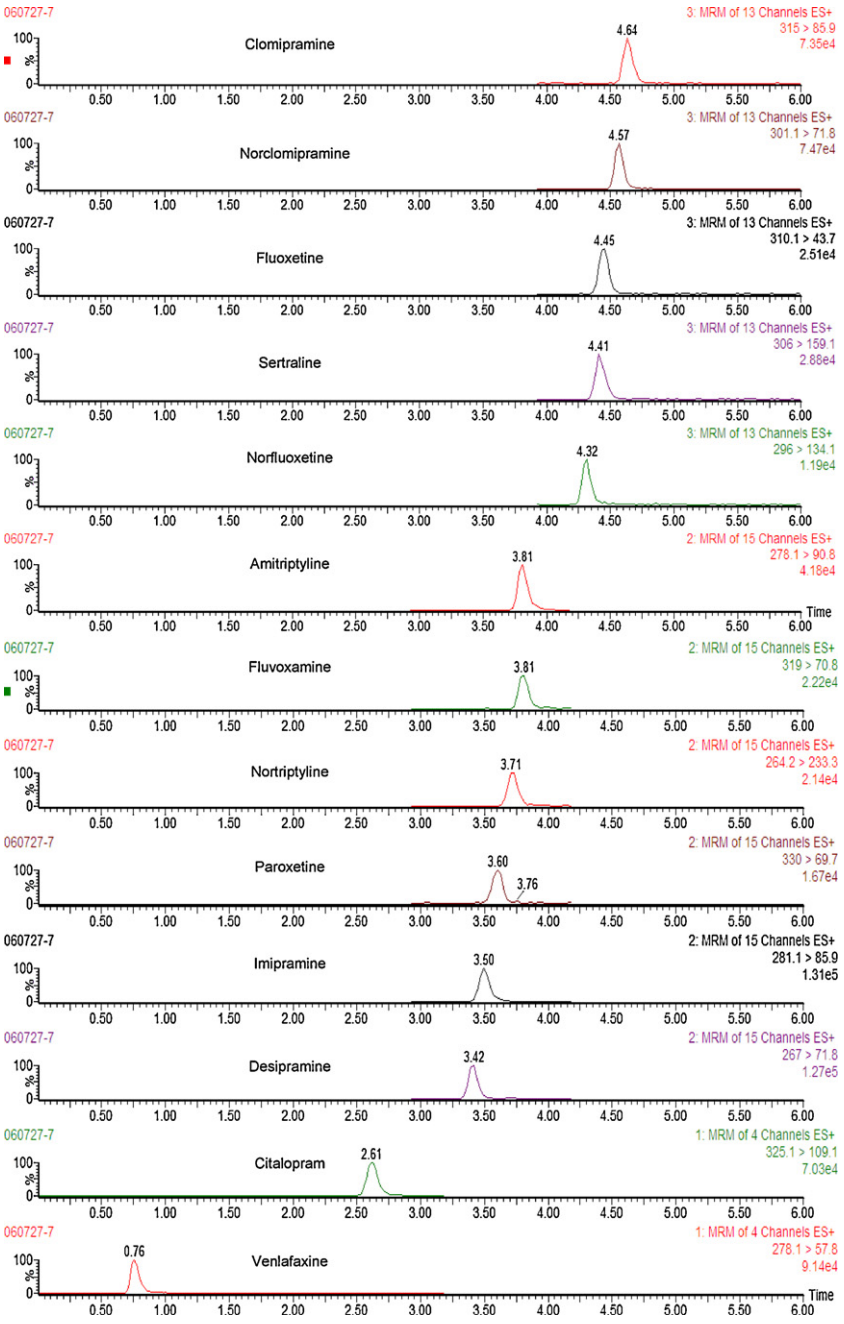

Fig. 2. Chromatograms of the quantifier transitions selected for each antidepressant in plasma at the LLOQ (2 ng/mL).

was found in the plasmatic free fraction ($C_{PL\text{-}FF}$) (11–53%), which could be due, among other factors, to some retention of the compound in the filter used to eliminate the plasmatic proteins. In all cases, oral fluid concentrations were higher than the plasmatic ones. This characteristic, also showed by other drugs [46,47] can be explained by the fact that venlafaxine is a weak base and tends to concentrate in oral fluid, which has a slightly lower pH than plasma.

For each patient, the correlation between C_{OF} vs. C_{PL} or $C_{PL\text{-}FF}$ in the four different days was analyzed by linear regression. Only in one out of the five patients coefficients of determination (r^2) >0.6 and 0.8 were found (for C_{PL} and $C_{PL\text{-}FF}$, respectively). CV in $R_{OF/PL}$ and $R_{OF/PL\text{-}FF}$ were between 24.2–69.6 and 12.9–58.8, respectively, indicating that a good correlation is not likely to be found (Table 7).

So, the results obtained in this study do not show a good correlation between venlafaxine levels in oral fluid and plasma or the plasmatic free fraction. Nevertheless, the small number of cases and the lack of homogeneity between them, do not allow making definitive conclusion in this point. This study should be extended, including more patients taking the same daily dose, and collecting a higher number of samples from each patient. Other parameters, such as the interval time between drug administration and samples collection should also be standardised. The main difficulty, nevertheless, is to persuade patients to participate in such a long and time-consuming study.

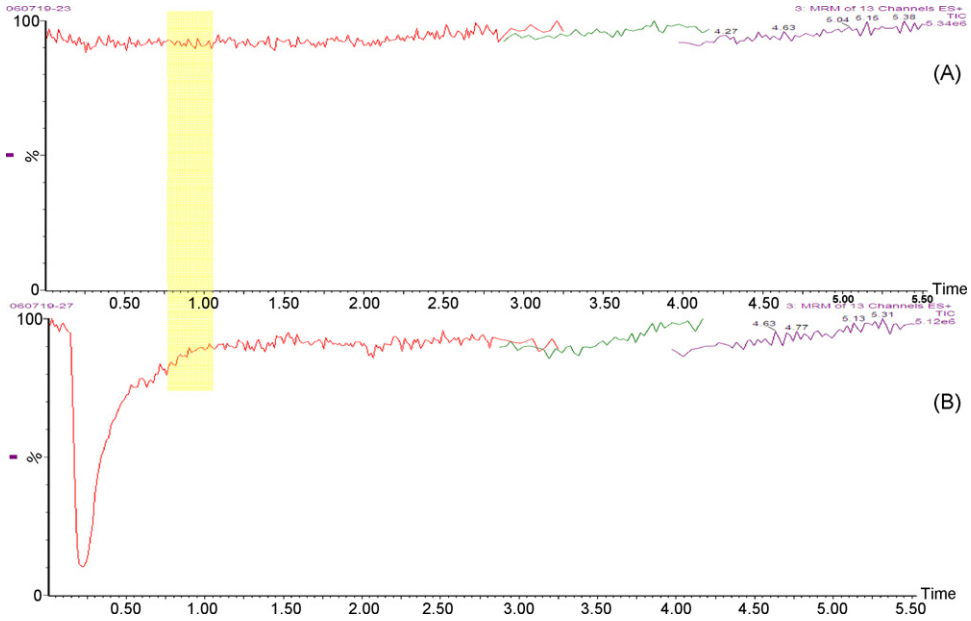

Fig. 3. TIC (total ion chromatogram) after the postcolumn infusion of a mixture of the antidepressants simultaneous to the injection of a mobile phase (A) and after the injection of an extracted blank oral fluid sample (B). The retention time for venlafaxine is highlighted.

Table 6
Oral fluid/plasma correlation study

Patient	Venlafaxine dose		Min.	Max.	Average	CV
1	150 mg, 1 dose/day	C_{PL}	74.7	125.7	97.3	21.8
		$C_{PL\text{-}FF}$	39.0	134.1	84.9	53.0
		C_{OF}	182.5	531.2	397.7	39.6
2	150 mg, 1 dose/day	C_{PL}	52.4	72.6	60.6	14.8
		$C_{PL\text{-}FF}$	37.2	46.2	42.1	11.2
		C_{OF}	285.6	404.7	323.9	16.8
3	75 mg, 1 dose/day	C_{PL}	17.5	25.7	22.5	15.6
		$C_{PL\text{-}FF}$	8.3	16.6	12.4	37.7
		C_{OF}	82.1	214.7	156.8	41.6
4	75 mg, 1 dose/day	C_{PL}	40.0	51.5	44.8	11.6
		$C_{PL\text{-}FF}$	13.8	20.9	17.0	17.3
		C_{OF}	144.4	289.2	215.7	27.4
5	150 mg, 1 dose/day	C_{PL}	25.1	44.9	33.8	24.7
		$C_{PL\text{-}FF}$	6.8	14.0	11.2	27.7
		C_{OF}	58.8	265.0	140.9	63.1

Venlafaxina results.

Table 7
Oral fluid/plasma correlation study

Patient		Min.	Max.	Average	CV	r^2
1	$R_{OF/PL}$	2.4	5.5	4.0	30.9	0.631
	$R_{OF/PL\text{-}FF}$	4.0	6.8	5.0	25.6	0.848
2	$R_{OF/PL}$	4.2	7.7	5.5	28.3	0.239
	$R_{OF/PL\text{-}FF}$	6.5	8.7	7.7	12.9	0.332
3	$R_{OF/PL}$	3.4	9.0	6.9	35.7	0.178
	$R_{OF/PL\text{-}FF}$	4.9	25.0	14.4	57.2	0.028
4	$R_{OF/PL}$	3.6	6.3	4.8	24.2	0.245
	$R_{OF/PL\text{-}FF}$	6.9	16.9	13.1	33.6	0.252
5	$R_{OF/PL}$	2.3	8.6	4.3	69.6	0.027
	$R_{OF/PL\text{-}FF}$	7.3	23.3	12.5	58.8	0.101

Venlafaxine results (II).

4. Conclusion

A rapid, selective and sensitive method has been developed for the analysis of the main antidepressants employed in the clinical practice in oral fluid and plasma. To our knowledge, until now this is the first LC–MS/MS method described for the simultaneous detection of these antidepressants in oral fluid. The method was applied to oral fluid and plasma samples from patients on venlafaxine treatment to assess the correlation between the concentrations of this compound in both matrices, concluding that a good correlation is not likely to be found. However, oral fluid samples could be employed in special situations, for example, to assess patient non-compliance.

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