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Analysis of the second generation antidepressant venlafaxine and its main active metabolite *O*-desmethylvenlafaxine in human plasma by HPLC with spectrofluorimetric detection

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Abstract

A high-performance liquid chromatographic method has been developed for the determination of the recent serotonin and norepinephrine reuptake inhibitor (SNRI) venlafaxine and its main active metabolite, *O*-desmethylvenlafaxine, in human plasma. Separation was obtained by using a reversed-phase column (C8, 150 × 4.6 mm I.D., 5 μm) and a mobile phase composed of 75% aqueous phosphate buffer containing triethylamine at pH 6.8 and 25% acetonitrile. Fluorescence detection was used, exciting at λ = 238 nm and monitoring the emission at λ = 300 nm. Citalopram was used as the internal standard. A careful pre-treatment of plasma samples was developed, using solid-phase extraction with C1 cartridges (100 mg, 1 mL). The limit of quantification (LOQ) was 1.0 ng mL^{-1} and the limit of detection (LOD) was 0.3 ng mL^{-1} for both analytes. The method was applied with success to plasma samples taken from patients undergoing treatment with venlafaxine. Precision data, as well as accuracy results, were satisfactory and no interference from other drugs was found. Hence, the method is suitable for therapeutic drug monitoring of venlafaxine and its main metabolite in depressed patients' plasma.

Keywords: Venlafaxine; High-performance liquid chromatography; Fluorescence detection; Human plasma; Solid-phase extraction; Therapeutic drug monitoring

1. Introduction

Venlafaxine (1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol, VLX, Fig. 1) is a second generation antidepressant drug [1]. It is one of the most potent selective serotonin and norepinephrine reuptake inhibitors (SNRI), and its therapeutic effects are attributed to this activity. When compared to the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine, VLX has demonstrated faster onset of action and better completeness of response [2]; VLX has also been used for patients who are non-responders to SSRIs or whose response to these drugs decreases over time [3]. VLX is administered as normal or sustained release capsules (Effexor, Efexor, Faxine) at doses ranging from 75 to 225 mg day^{-1}. For severe depression in hospitalised patients the dose can reach 375 mg day^{-1} [4]. Maximum plasma levels are reached after about 2 h and plasma half-life is 4–9 h [5]. For this reason, an extended-release (XR) formulation has been developed, suitable for once daily dosing [6]. VLX is mainly metabolised in the liver by cytochrome P450 (CYP) enzymes [7] and the most abundant metabolite is *O*-desmethylvenlafaxine (ODV, Fig. 1). ODV is pharmacologically active and significantly contributes to the therapeutic effect of VLX since it is found in plasma at high concentrations [8] and has a long half-life (11–13 h) [9]. Therapeutic plasma levels of VLX usually range from 30 to 200 ng mL^{-1}, while the corresponding levels of ODV are in the 50–500 ng mL^{-1} range [10]. While the pharmacotoxicological profile of VLX is surely much more favourable than that of traditional tricyclic antidepressants [11], treatment with VLX can still cause several side effects regarding the

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nervous system (vertigo, dry mouth, insomnia, nervousness, somnolence), the gastrointestinal system (anorexia, constipation, nausea), the cardiovascular system (hypertension, vasodilation, palpitations) and the genitourinary system (impotence, anorgasmia) [5]. Therefore, the great importance of therapeutic drug monitoring (TDM) in patients undergoing treatment with VLX can easily be understood. TDM is currently considered a powerful tool for therapy optimisation and personalisation [12–15]. In psychiatric clinics, it has until now mostly been applied to antipsychotic therapy. However, in the last few years, TDM is acquiring more importance in antidepressant therapy [16], especially when metabolic anomalies or low compliance are suspected, or in case of polypharmacy.

Several methods can be found in the literature for the determination of therapeutic levels of VLX and ODV in human plasma or serum [17–25]. Most of them use HPLC coupled to spectrofluorimetric [21,23], spectrophotometric [18,22,24], electrochemical (coulometric) [20] or mass spectrometric [17] detection. Capillary electrophoresis with UV detection has been used as well [19,24]. All cited methods carry out sample pretreatment by liquid–liquid extraction. This has the disadvantage of using relatively high volumes of polluting and dangerous organic solvents and of being quite labour intensive and time-consuming. Furthermore, several methods [18,19,21–24] have limits of quantification (LOQ) values in the 5–50 ng mL^{-1} range. While they can determine therapeutic levels of VLX and ODV, they are not suitable for the TDM of patients receiving low doses or in cases of low compliance. Other methods exploit expensive instrumentation such as HPLC–MS [17] or not easily available instrumentation such as the coulometric detector [20].

Thus, the aim of this work was the development and validation of a new analytical method for the analysis of VLX and ODV in patient plasma. It had to reach high sensitivity and to have a wide linearity range while using easily available, affordable and reliable instrumentation.

2. Experimental

2.1. Chemicals and solutions

Venlafaxine (99.5% purity) and *O*-desmethylvenlafaxine (99.1% purity) were kindly provided by Wyeth (Madison, NJ, USA). Citalopram (1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile, Fig. 1), used as the Internal Standard (IS), was kindly donated by Lundbeck A/S (Copenhagen, Denmark). Acetonitrile and methanol (HPLC grade, ≥99.8%), 85% (w/w) phosphoric acid and monobasic sodium phosphate (pure for analysis, ≥99%) were purchased from Carlo Erba (Milan, Italy). Triethylamine pure for analysis (≥99.5%) was purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, USA).

Stock solutions of the analytes (1 mg mL^{-1}) were prepared by dissolving suitable amounts of each pure substance in methanol. Standard solutions were obtained by diluting stock solutions with the mobile phase. Stock solutions were stable for at least 2 months when stored at −20 °C (as assessed by HPLC assays); standard solutions were prepared fresh every day.

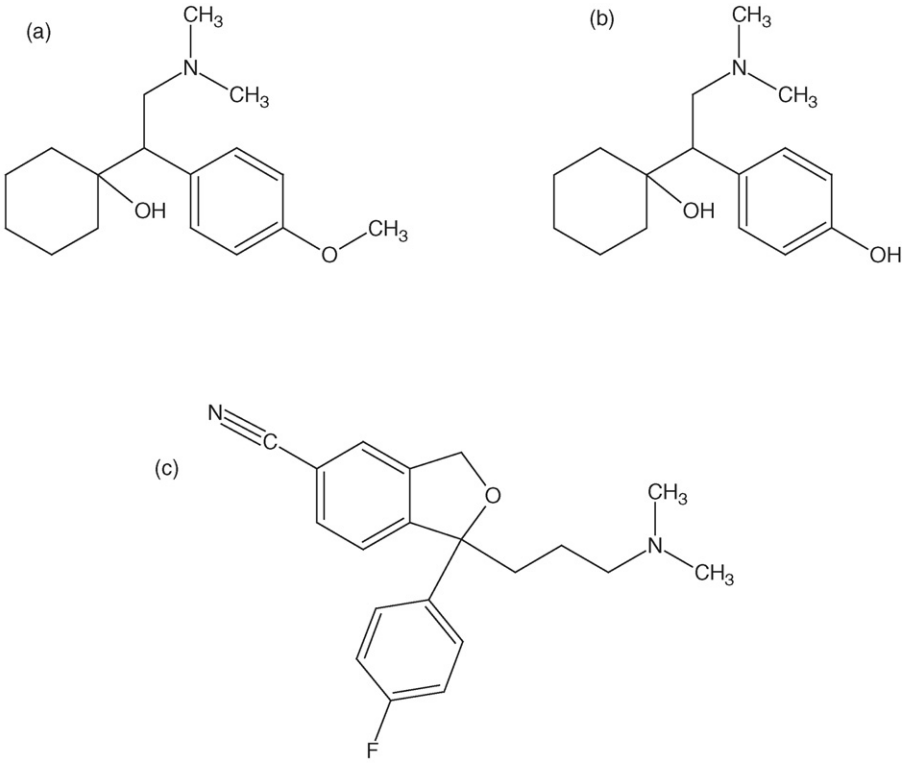

Fig. 1. Chemical structures of (a) venlafaxine (VLX), (b) *O*-desmethylvenlafaxine (ODV) and (c) citalopram (IS).

Table 1
Flow rate gradient parameters

Start time (min)	End time (min)	Flow rate (mL min^{-1})
0.0	3.5	1.0
3.5	4.0	1.0–2.0, linear gradient
4.0	15.0	2.0
15.0	15.5	2.0–1.0, linear gradient

2.2. Instrumentation and chromatographic conditions

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-2089 PLUS chromatographic pump and a Jasco FP-2020 spectrofluorimetric detector set at $\lambda_{exc} = 238$ nm, $\lambda_{em} = 300$ nm.

Separations were obtained on an Agilent (Waldbronn, Germany) Zorbax C8 reversed-phase column (150 × 4.6 mm I.D., 5 μm) kept at room temperature. The mobile phase was composed of a mixture of acetonitrile (25%, v/v) and a pH 6.8, 40 mM phosphate buffer containing 0.25% (v/v) triethylamine (75%, v/v). The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic bath. A flow rate gradient was programmed during the analytical run; specific parameters are reported in Table 1. The injections were carried out through a 20 μL loop. Data processing was handled by means of a Jasco Borwin 3.0 software.

Solid-phase extraction (SPE) was carried out on a Vac Elut (Varian) apparatus.

A Crison (Barcelona, Spain) Basic 20 pH meter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Sample collection and preparation

The blood samples were collected from patients admitted to the psychiatric ward of the University of Parma (Italy), subjected to therapy with VLX for at least 2 weeks at constant daily doses. Blood samples were usually drawn 12 h after the last drug administration. Blood was stored in glass tubes containing EDTA as the anticoagulant, then centrifuged (within 2 h from collection) at 1400 × *g* for 15 min; the supernatant (plasma) was then transferred to polypropylene test tubes and stored at −20 °C until HPLC analysis. "Blank" plasma was obtained in the same way from blood drawn from healthy volunteers not subjected to any pharmacological treatment.

The solid-phase extraction procedure was carried out on Varian (Walnut Creek, USA) BondElut C1 cartridges (100 mg, 1 mL). Cartridges were activated by passing 1 mL of methanol through the cartridge five times and then conditioned by passing 1 mL of ultrapure water five times. To 250 μL of plasma, 500 μL of ultrapure water and 50 μL of IS working solution were added and the resulting mixture was loaded onto a previously conditioned cartridge. The cartridge was then washed twice with 1 mL of ultrapure water, once with 1 mL of water/methanol mixture (70/30, v/v) and once with 50 μL of methanol. The analytes were then eluted with 1 mL of methanol. The eluate was dried under vacuum (rotary evaporator), redissolved with 250 μL of mobile phase, and then injected into the HPLC system.

2.4. Method validation

2.4.1. Calibration curves

Aliquots of 50 μL of analyte standard solutions at seven different concentrations containing the IS at a constant concentration were added to 250 μL of blank plasma. The resulting mixture was subjected to the previously described SPE procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios obtained (dimensionless numbers) were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were set up by means of the least-square method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP [26] guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.4.2. Extraction yield (absolute recovery)

The procedure was the same as that described under Section 2.4.1, except the points were at three different concentrations, corresponding to the upper limit, lower limit and middle point of each calibration curve. The analyte/IS peak area ratios were compared to those obtained by injecting standard solutions at the same theoretical concentrations, and the extraction yield values were calculated as follows:

$$100 \left[\frac{(\text{analyte/IS peak area ratio})_{\text{spiked blank}}}{(\text{analyte/IS peak area ratio})_{\text{corresponding standard}}} \right].$$

2.4.3. Precision

The assays described under Section 2.4.2 were repeated six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as RSD% values.

2.4.4. Selectivity

Blank plasma samples from six different volunteers were subjected to the SPE procedure and injected into the HPLC. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was, that there is no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different drugs active on the central nervous system were injected at concentrations higher than the respective therapeutic levels; if the resulting chromatograms contained any interfering peak, the potentially interfering compounds were subjected to SPE and injected to ascertain if they were extracted.

2.4.5. Accuracy

Accuracy was evaluated by means of recovery assays. The assays described under Section 2.4.2 were carried out adding standard solutions of the analytes and the IS to real plasma samples taken from depressed patients subjected to therapy with VLX. The assays were repeated six times during the same day to obtain mean recovery and RSD% data.

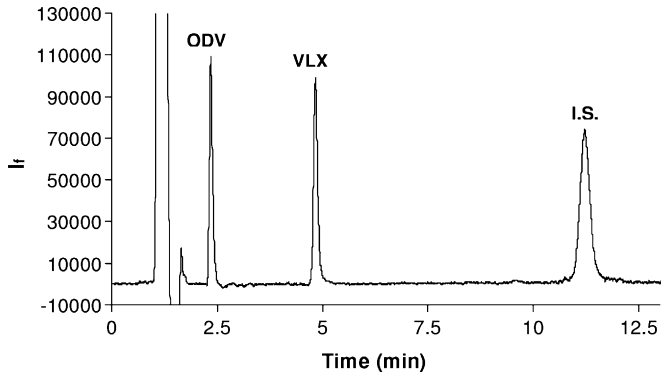

Fig. 2. Chromatogram of a standard solution containing 50 ng mL^{-1} of VLX, 50 ng mL^{-1} of ODV and 100 ng mL^{-1} of the IS.

3. Results and discussion

3.1. Choice of the experimental conditions

Both VLX and ODV are natively fluorescent molecules. The fluorescence spectra of the analytes in a water/acetonitrile mixture exhibit an emission maximum at 300 nm when exciting at 238 nm; thus, these wavelengths were chosen for the subsequent assays.

The mobile phase was initially composed of acetonitrile and pH 6.0 phosphate buffer (50/50, v/v). However, using this mobile phase and a C8 (Zorbax, 150 × 4.6 mm I.D., 5 μm) stationary phase, ODV was not retained and VLX was only weakly retained. Thus, lower percentages of acetonitrile (40–20%) were tested. Good results were obtained with all acetonitrile percentages lower than 30%. In order to obtain the shortest possible retention times, 25% acetonitrile was chosen. Since the analyte peaks were quite asymmetric, 0.25% triethylamine was added to the mobile phase, which was enough to obtain good peak shapes. Some fluorescent compounds such as fluorescein, dichlorofluorescein, eosin, mianserin and paroxetine were tested as possible ISs. However, they were poorly retained or had low fluorescence emission. On the contrary, citalopram gave good fluorescence emission intensity but was strongly retained. Thus, a flow rate gradient was programmed, which increases the rate from 1.0 to 2.0 mL min^{-1} after the elution of ODV. The chromatogram of a standard solution containing VLX, ODV and the IS is reported in Fig. 2. As can be seen, the peaks are neat, symmetric and well separated and the flow rate changes do not distort in any way the baseline appearance.

3.2. Analysis of standard solutions

Seven-point calibration curves with a range of 1–1000 ng mL^{-1} were set up for both analytes. Good linearity ($r^2 > 0.9995$) was obtained for both compounds, with a limit of quantification value of 1.0 ng mL^{-1} and a limit of detection value of 0.3 ng mL^{-1}.

Precision was evaluated at three concentrations (1, 200 and 800 ng mL^{-1}). RSD values for repeatability (intraday precision) were lower than 1.8% for VLX and lower than 2.2% for ODV. RSD values for intermediate precision (interday precision) were lower than 3.6% for VLX and lower than 4.4% for ODV.

3.3. Development of the solid-phase extraction procedure

SPE was chosen as the sample pre-treatment procedure because it confers high selectivity to the method and allows to obtain good sample purification and extraction yields. Different kinds of sorbents were tried, such as hydrophilic–lipophilic balance (HLB), cyanopropyl (CN), phenyl (PH), C1, C2 and C8. HLB and CN sorbents gave low extraction yields of the analytes, while the PH sorbent was not sufficiently selective. C1, C2 and C8 sorbents gave good sample purification and extraction yields of the analytes; however, C2 and C8 cartridges did not sufficiently retain the IS. Thus, the C1 sorbent was chosen for the SPE procedure. The washing step was initially carried out with water only, but in order to obtain better purification, a washing step using a methanol/water (30%, v/v) mixture was added. Another washing step with a small volume (50 μL) of methanol eluted other interferents but not the analytes. The latter were eluted with 1 mL of methanol.

It should be noted that other published HPLC methods with fluorimetric detection [21,23] use liquid–liquid extraction procedures, which are more time-consuming (up to an entire night) than the proposed SPE procedure and also require higher volumes of sample (1 mL instead of 250 μL).

Using this SPE procedure, good extraction yields of the analytes and the IS were obtained, while eliminating endogenous interference. These results can be seen in Fig. 3. Fig. 3a shows the chromatogram of a blank plasma sample after SPE, while Fig. 3b shows the chromatogram of the same blank plasma sample spiked with a known amount of the analytes and the IS. No interference can be detected near the retention times of the compounds of interest. Furthermore, peak shapes and resolution are good and similar to those obtained by injecting standard solutions.

Table 2
Linearity parameters

Compound	Linearity range (ng mL^{-1})	Equation coefficients, $y = a + bx$[a]		r^2	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
		a	b			
VLX	1–1000	−0.0052	0.0089	0.9990	1.0	0.3
ODV	1–1000	−0.0027	0.0093	0.9990	1.0	0.3

[a] y = analyte/IS peak area ratio; x = analyte concentration (ng mL^{-1}).

Table 3
Precision and extraction yield results

Compound	Concentration (ng mL^{-1})	Repeatability, RSD%[a]	Intermediate precision, RSD%[a]	Extraction yield (%)[a]
VLX	1	4.6	4.7	93
	200	2.5	4.3	94
	800	1.8	4.1	96
ODV	1	4.2	4.5	94
	200	2.1	3.8	95
	800	1.2	3.6	97
IS	100	3.1	4.5	97

[a] $n = 6$.

3.4. Method validation

Satisfactory linearity ($r^2 \geq 0.9990$) was obtained for both analytes in the 1–1000 ng mL^{-1} concentration range, with values of LOQ and LOD equal to 1.0 and 0.3 ng mL^{-1}, respectively. Complete linearity parameters can be found in Table 2.

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma spiked with analyte concentrations corresponding to the lower, middle and upper limit of the calibration curves (i.e., 1, 200 and 800 ng mL^{-1}). The results of these assays are reported in Table 3. Mean extraction yields were always higher than 92% for VLX and 93% for ODV (97% for the IS). Precision results were also satisfactory. RSD values for repeatability were always lower than 4.7% for VLX and 4.3% for ODV (3.1% for the IS); RSD values for intermediate precision were lower than 4.8% (4.5% for the IS).

Selectivity was evaluated by injecting standard solutions of several drugs commonly coadministered during psychiatric therapy (for example, other antidepressants, anxiolytics–hypnotics, antipsychotics and antiepileptics). The complete list of these

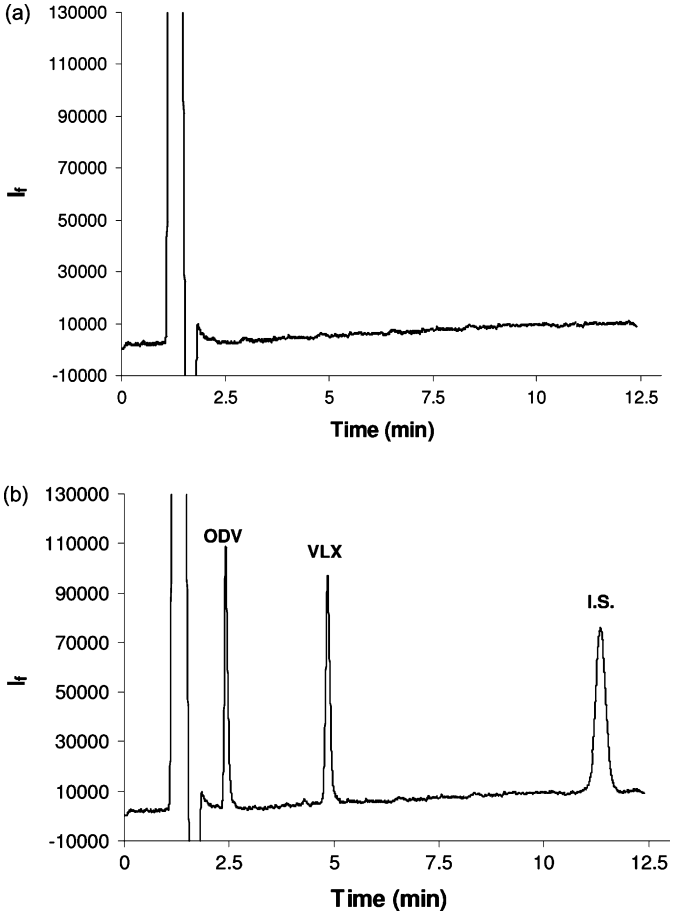

Fig. 3. Chromatograms of (a) a blank plasma sample from a healthy volunteer and (b) the same blank plasma sample spiked with 50 ng mL^{-1} of VLX, 50 ng mL^{-1} of ODV, and 100 ng mL^{-1} of the IS.

Table 4
Drugs tested for interference

Therapeutic class	Compound	Retention time (min)
Analytes	ODV	2.4
	VLX	4.8
	Citalopram (IS)	11.4
Antidepressants	Amitriptyline	n.d.[a]
	Duloxetine	n.d.
	Fluoxetine	4.8
	Imipramine	n.d.
	Maprotiline	n.d.
	Mirtazapine	n.d.
	Paroxetine	n.r.[b]
	Sertraline	n.d.
	Trazodone	n.d.
Antipsychotics	Clotiapine	n.d.
	Clozapine	n.d.
	Fluphenazine	n.d.
	Haloperidol	n.d.
	Levomepromazine	n.d.
	Olanzapine	n.d.
	Quetiapine	n.d.
	Risperidone	n.d.
Anxiolytics–hypnotics	Brotizolam	n.d.
	Clonazepam	n.d.
	Diazepam	n.d.
	Flurazepam	n.d.
	Lorazepam	n.d.
Antiepileptics	Carbamazepine	n.d.
	Lamotrigine	n.d.
	Valproate	n.d.
Abuse drugs	Buprenorphine	n.d.
	MDMA	n.d.
	Δ^9-Tetrahydrocannabinol	n.d.

[a] n.d. = not detected within a 30-min run.
[b] n.r. = not retained.

drugs is reported in Table 4. As can be seen, only one of the 29 tested drugs (fluoxetine) is retained and detected within a 30-min run. However, it does not interfere with the determination of VLX and ODV. Furthermore, six blank plasma samples were injected after SPE and none of them produced peaks from endogenous compounds which could interfere with the determination.

3.5. Analysis of patient plasma samples

Having thus validated the method, it was applied to the analysis of plasma samples from patients admitted to the psychiatric ward of the University of Parma (Italy) undergoing therapy with VLX (Efexor®). As examples, the chromatograms of plasma samples from patients taking 75 mg day^{-1} and 150 mg day^{-1} of VLX are shown in Fig. 4a and b, respectively. Analyte concentrations found in these real samples were the following: patient 1 (75 mg day^{-1}), 38 ng mL^{-1} of VLX and 53 ng mL^{-1} of ODV; patient 2 (150 mg day^{-1}), 70 ng mL^{-1} of VLX and 150 ng mL^{-1} of ODV. Peak shapes and resolution are very similar to those obtained using spiked blank plasma, and no interference from endogenous or hexogenous compounds is apparent. In fact, in addition to VLX, the first patient was taking haloperidol and flurazepam and the second patient was taking clozapine. As expected, none of the coadministered drugs interfered with the determination, thus confirming the high selectivity of the method.

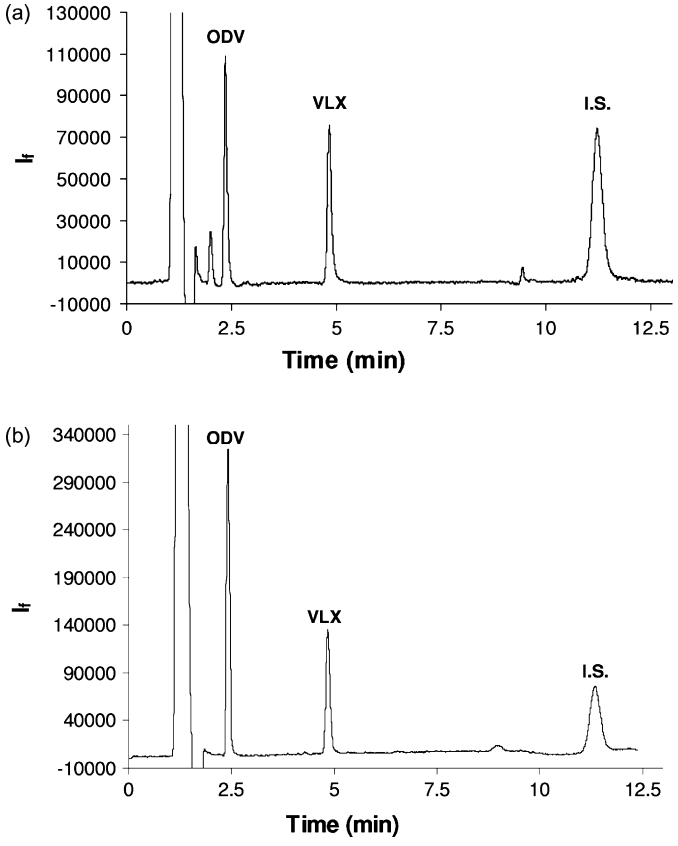

Fig. 4. Chromatograms of (a) a plasma sample from a patient subjected to treatment with 75 mg day^{-1} of VLX, as well as haloperidol and flurazepam; (b) a plasma sample from a patient subjected to treatment with 150 mg day^{-1} of VLX, as well as clozapine.

Table 5
Accuracy results

Compound	Analyte concentration added (ng mL^{-1})	Recovery ± SD
VLX	1	98 ± 1.4
	200	99 ± 1.3
	400	100 ± 1.2
ODV	1	97 ± 1.6
	200	98 ± 1.5
	400	99 ± 1.3

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations (1, 200 and 400 ng mL^{-1}) and of the IS at a constant concentration (100 ng mL^{-1}) were added to plasma samples containing known amounts of VLX and ODV (i.e., samples which had been already analysed). Then, the recovery of the analytes, as well as the standard deviation of the assays, was calculated. The results thus obtained are reported in Table 5. Mean recovery values were 99% for VLX and 98% for ODV.

4. Conclusion

The HPLC method with fluorimetric detection presented here for the simultaneous analysis of VLX and ODV is simple, sensitive and reliable.

The SPE procedure implemented for the sample pretreatment, based on C1 cartridges, gives good extraction yields (>92% for both analytes) and satisfactory precision (RSD% < 4.8%). The fluorimetric detection and the SPE procedure together make the method selective: neither endogenous compound nor any of the central nervous system drugs tested has caused any interference in the therapeutic monitoring of VLX and ODV in depressed patient plasma. The use of SPE poses several advantages with respect to the liquid–liquid extraction procedures used by other authors [17–25]. In fact, the SPE procedures are more feasible, faster and require lower volumes of organic solvents. Extraction yield values also are higher than those reported by other authors [20] and precision values are better than those reported in most other papers [17,18,20–24]. The proposed method is also advantageous because it has high accuracy and a wide linearity range. This allows the determination of the analytes not only at therapeutic doses but also in overdose cases and when administered at sub-therapeutic doses (e.g., scarce compliance). Other methods are not able to reliably determine the analytes at these non-typical levels [18,19,21–24].

When compared to other HPLC–fluorescence methods [21,23], the proposed method has much higher extraction yield results with respect to ODV (94–97% versus 52–68%) and also better precision (RSD < 4.8% versus RSD < 14% [21]) and lower LOQ values (1 ng mL^{-1} versus 14 ng mL^{-1} [23]); furthermore, the proposed SPE procedure is less polluting and time-consuming than the liquid–liquid extraction procedures used in these methods (which can require up to 12 h of overnight

freezing) and uses lower volumes of plasma (250 μL versus 1 mL).

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