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Rapid determination of venlafaxine and O-desmethylvenlafaxine in human plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

A rapid and sensitive high-performance liquid chromatographic technique for simultaneous measurement of plasma venlafaxine (VEN) and its active metabolite O-desmethylvenlafaxine (ODV) is described. The process begins with the extraction of VEN and ODV, with maprotiline (MAP) as internal standard, from human plasma into an intermediate organic mixture of hexane–isoamyl alcohol and finally into an aqueous solution of 0.05% phosphoric acid. Isocratic separation of VEN, ODV and MAP is carried out by utilizing a reversed-phase butyl-bonded column (C_4/E) with mobile phase consisting of acetonitrile and 40 m*M* phosphate buffer, pH 6.8 (50:50, v/v). Detection of VEN, ODV and MAP is done by mean of fluorimetry with excitation and emission wavelengths set at 276 and 598 nm, respectively. As low as 1.0 ng/ml VEN is detectable; while the limit of detection for ODV is 5 ng/ml. C.V. (%) of intra-day samples for both VEN and ODV are less than 10% at three concentrations tested (10.0, 50.0, 100.0 ng/ml). Similarly, over the same nominal concentrations, the precision of inter-day (5 days) samples also results in C.V. (%) smaller than 10% for both compounds, except for ODV measured at 10 ng/ml (C.V.$<$15%). Approximately, 100% VEN can be extracted from plasma; whereas, for ODV the recovery rate is nearly 70%. This present method is rapid, sensitive, accurate and simple for routine clinical monitoring of plasma VEN and its major metabolite ODV.

Keywords: Venlafaxine; Desmethylvenlafaxine; Antidepressants

1. Introduction

Venlafaxine hydrochloride (VEN), 1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl] cyclohexanol hydrochloride, belongs to a new class of non-tricyclic antidepressants undergoing extensive clinical investigations. Its structure is distinct from that of the traditional tricyclics yet it exerts its antidepressant effects by inhibiting the same neuronal uptake mechanisms of serotonin, norepinephrine, and to lesser extent of dopamine [1–3]. It lacks many adverse side effects normally associated with the administration of tricyclics [1,3,4].

VEN has several metabolites (Fig. 1), one of which is biologically active. About 1% of VEN is desmethylated to N-desmethylvenlafaxine (NDV), 1-[2-(methylamino)-1-(4-methoxyphenyl)ethyl] cyclohexanol; 16% becomes N,O-didesmethylvenlafaxine (NODV), 4-[2-(methylamino)-1-(1-hydroxycyclo-

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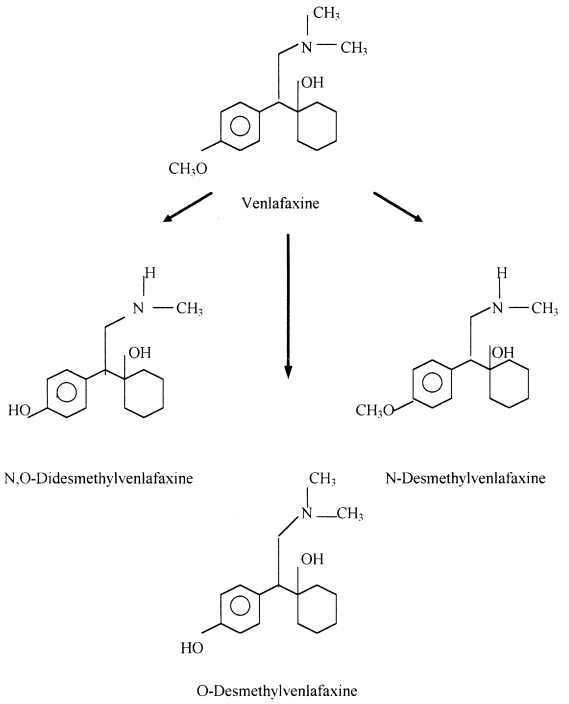

Fig. 1. Molecular structures of venlafaxine and its metabolites.

hexyl)ethyl]phenol; and 56% is metabolized to O-desmethylvenlafaxine (ODV), 4-[2-(dimethylamino)-1-(1-hydroxycyclohexyl)ethyl]phenol which, unlike the others, has an activity profile on monoamine transporters similar to VEN [5,6]. Therefore, both VEN and ODV levels in plasma and/or urine are important pharmacokinetic and pharmacodynamic parameters in assessing their efficacy. Currently there are two published high-performance liquid chromatography (HPLC) procedures for monitoring plasma levels of VEN and ODV [7,8]; however, neither utilizes fluorimetry as the means of detection. Taking advantage of VENs fluorescence properties, this report presents a rapid, accurate and sensitive reversed-phase HPLC method for simultaneous determination of VEN and ODV in human plasma.

An application of this method has been successfully implemented in a clinical study investigating the effect of VEN on the metabolism of imipramine. Chromatograms and mean steady state plasma levels of VEN and ODV of subject samples are included here to demonstrate possible interference with other metabolites and ultimately the suitability of this method for measuring VEN and ODV in human plasma.

2. Experimental

2.1. Materials

VEN and ODV (free base) were obtained from Wyeth–Ayerst (West Chester, PA, USA) and the internal standard, maprotiline hydrochloride (MAP), was obtained from Ciba Geigy (Basel, Switzerland). Sodium bicarbonate (ACS-grade), sodium phosphate monobasic (ACS-grade), and dimethyl sulfoxide (ACS-grade) were from Sigma (St. Louis, MO, USA). Hexane (HPLC-grade), methanol (HPLC-grade), acetonitrile (HPLC-grade), *o*-phosphoric acid (HPLC-grade), and isoamyl alcohol (certified-grade) were from Fisher Scientific (Tustin, CA, USA). Distilled, Millipore quality [prefiltration (MF-Lifegard II cartridge)], organic absorption (Super-C cartridge), deionization (ion-exchange cartridge), and Millipore filtration (Millitube MF) (Millipore, Bedford, MA, USA) was used throughout all procedures. A LS50B spectrofluorometer (Perkin Elmer, Beaconsfield, UK) was used to scan for optimal excitation and emission wavelengths of all drugs involved. A horizontal, single-speed shaker (300 rpm) (Eberbach, Ann Arbor, MI, USA) was utilized in the plasma extraction procedures. The HPLC system consisted of the following components: from Waters Associates (Milford, MA, USA) a Waters 600E solvent delivery pump, a Waters Wisp 712 Model automatic injector, and a Waters 470 fluorescence detector; from Spectra-Physics (San Jose, CA, USA) a SP4290 integrator; and from MetaChem Technologies (Torrance, CA, USA) a C_4/E column (150×4.6 mm, 5 μm) fitted with a C_4/E guard column (13×4.3 mm, 5 μm).

2.2. Methods

2.2.1. Reagent preparation

Stock solutions of VEN and MAP (1 mg/ml) were prepared in water and methanol, respectively. Stock ODV (100 μg/ml) was made by overnight stirring in an aqueous solution containing 10% (v/v) each of methanol and dimethyl sulfoxide. Serial dilution of

stock drug solutions were used for standard curves. HPLC mobile phase consisting (50:50 v/v) of acetonitrile and sodium phosphate buffer (40 m*M*, pH 6.8) was premixed, filtered through a 0.2 μm PTFE filter, and degassed by sonication before use. Other solutions used were: 0.7 *M* sodium bicarbonate buffer (pH 9.7), 0.05% (v/v) *o*-phosphoric acid and 7.5% (v/v) isoamyl alcohol in hexane.

Control plasma was prepared from pooled platelet-rich-plasma supplied by the Red Cross (Los Angeles, CA, USA). After removal of platelets and residual blood cells by centrifugation at 1500 *g* (30 min, room temperature), cell-free plasma was stored in a −20°C freezer until use. On the day of experiment, control plasma was thawed and again centrifuged at 1500 *g* (20 min, room temperature) to remove precipitates [9]. Control plasma was spiked with drug standards prior to organic extraction.

2.2.2. Extraction procedure

For organic extraction 50 μl MAP (final concentration 100 ng/ml) and 25 μl each of VEN and ODV standards (final concentrations 0, 1, 5, 10, 50, 100 ng/ml, etc.) were added into polypropylene test tubes (75×12 mm size). Next, 1 ml of control plasma was added followed by 400 μl of the basic bicarbonate buffer (0.7 *M*, pH 9.7). Samples were then vortexed for 1 min to basify the solution and extracted by the addition of 1.5 ml of 7.5% isoamyl alcohol–hexane followed by shaking at 300 rpm for 30 min, room temperature. Centrifugation at 1500 *g*, 15 min, room temperature allowed clear separation of organic from aqueous phase. After overnight freezing of samples at −20°C, the top organic layer was decanted into a 15 ml conical polypropylene tube already containing 200 μl of 0.05% *o*-phosphoric acid. Samples were then back-extracted into the acidic aqueous phase by shaking vigorously at 300 rpm for 45 min, room temperature. After phase separation by centrifugation, as described above, and subsequent aspiration of the top organic layer, the aqueous phase containing the extracted analytes was ready for analysis by HPLC [10].

2.2.3. HPLC conditions

Samples (100 μl) were injected using the 712 Wisp automation system (one sample every 20 min) under isocratic conditions (50:50, v/v acetonitrile to 40 m*M* sodium phosphate, pH 6.8; flow-rate 1.5 ml/min). Fluorescence measurements were done at 276 nm excitation and 598 nm emission wavelengths, with spectral bandwidth and electronic filter time constant set at 18 nm and 1.5 s, respectively. The column was kept at room temperature throughout all analyses.

2.2.4. Selectivity, sensitivity and linearity

To evaluate potential interference due to endogenous substances co-extracted during the process, we randomly chose Red Cross plasma packets for experimentation. To test for maximum sensitivity of this assay, control plasma was spiked with low drug standard concentrations (final amounts of 0.25, 0.5, 1.0, 2.5, 5.0 ng/ml). Conversely, to establish the range of linearity between drug standard concentration and fluorescence intensity the standard concentrations of 0, 1, 5, 10, 50, 100, 500, 1000, 2000 ng/ml were used. For calibration 0, 5, 10, 25, 50, 100 and 500 mg/ml concentrations were used.

2.2.5. Recovery, precision and accuracy

By comparing the peak areas obtained from the extracted samples spiked with known amounts of drugs to those obtained from the pure compounds of the same concentrations, the extraction recoveries (% recovery) of VEN and ODV were determined. Accuracy of the assay was derived by calculating the percent difference (% difference) between the amount of VEN and ODV added and found. To evaluate the methods precision, intra-day and inter-day (days 1, 2, 3, 4 and 5) measurements of VEN and ODV were completed with computation of the coefficient of variation (C.V.%) for replicate samples ($n=5$ for intra-day, $n=25$ for inter-day) using concentrations 10, 50, 100 ng/ml. Both intra and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis.

3. Results

3.1. Fluorescence scanning

The resulting profile of fluorescence scanning for optimal excitation and emission wavelengths for VEN, ODV and MAP are shown in Fig. 2. Besides

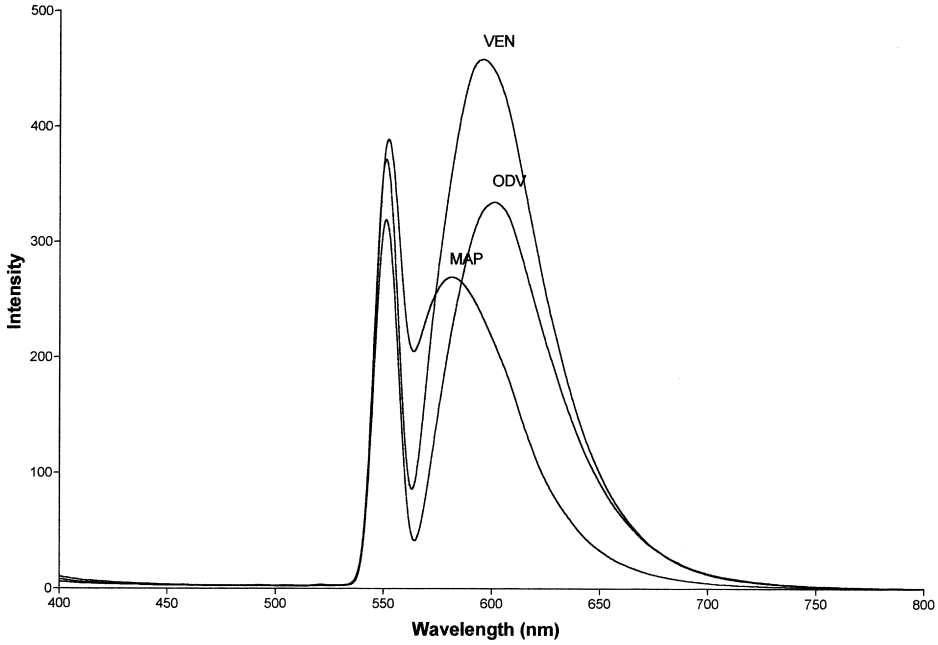

Fig. 2. Emission spectra [300–800] of (1) VEN, (2) ODV and (3) MAP when excitation wavelength is 276 nm.

the light scattering peak of the excitation beam at 552 nm, optimal excitation and emission peaks for VEN, ODV and MAP derived from these profiles were 276 and 598 nm, 277 and 605 nm and 272 and 585 nm, respectively. The excitation and emission wavelengths for VEN were used throughout this procedure.

3.2. Selectivity, sensitivity and linearity

Fig. 3a and Fig. 3b illustrate fluorescence–HPLC chromatograms of a blank and a 10 ng/ml-spiked plasma sample (Red Cross). Fig. 3c depicts a real subject blank spiked with 25 ng/ml maprotiline; while Fig. 3d shows that of a subject plasma sample collected 1.5 h after administration of oral venlafaxine. Under the conditions specified, relative retention times for VEN and ODV to MAP were 0.650 and 0.405, respectively. Chromatograms of blank plasma from both Red Cross and subject (Fig. 3a,c) were free of endogenous interfering peaks. The total eluting time was less than 20 min. Table 1 provides the sensitivity level achieved by this method. Concentrations as low as 1 ng/ml VEN and 5 ng/ml ODV were detectable; the C.V. was less than 10% for both cases. Table 2 summarizes linear regression statistics for representative standard curves. Regression lines relating VEN and ODV standard concentrations to peak area ratios were calculated using simple regression analysis. For both VEN and ODV the method was linear between 1.0 and 2000 ng/ml. Linear parameters for a typical standard curve are: (standard curve 1) correlation coefficients, $r^2 > 0.993$; slope $= 0.0253$, y-intercept $= -0.011$ (Table 2).

3.3. Recovery, precision and accuracy

Percent recoveries of compounds from spiked plasma following the extraction procedure are listed in Table 3. For VEN, the recovery was approximately 100% over four concentrations tested (5, 10, 50, 100 ng/ml); whereas, for ODV the recovery was 68%. For MAP, about 53% of the spiked 100 ng/ml was recovered. Evaluation of both the accuracy and precision of the method is summarized in Table 4. Results of the intra-day precision, assessed by calculating the C.V. for the analysis of replicates ($n=5$) at three nominal concentrations (10, 50, 100 ng/ml) of both VEN and ODV reveal a C.V.$<$10% for all cases. Likewise, inter-day (days 1, 2, 3, 4 and 5)

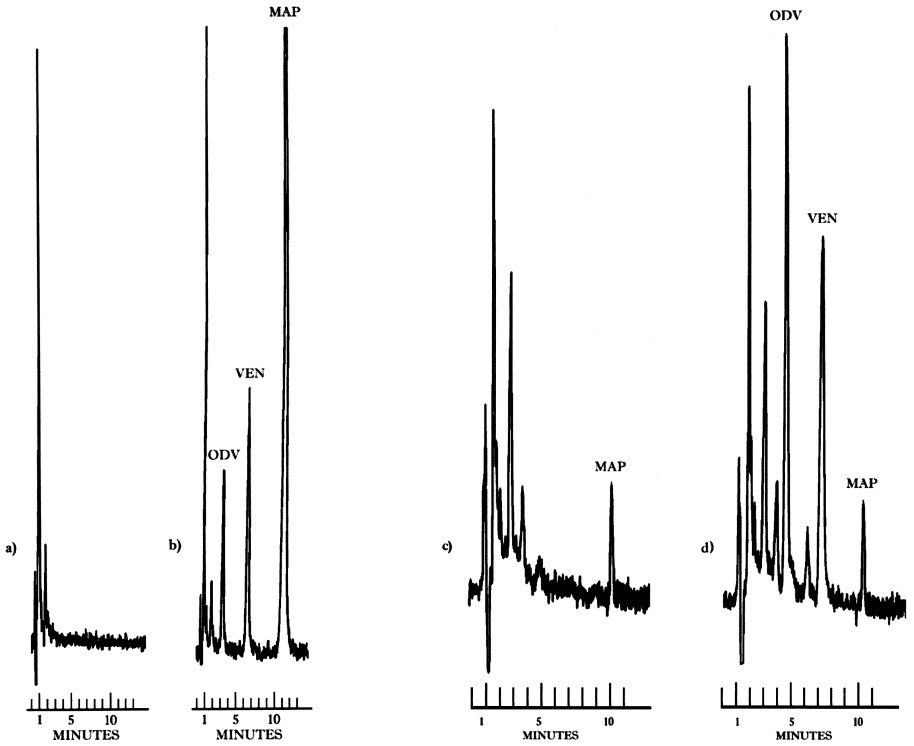

Fig. 3. HPLC chromatograms of (a) Red Cross blank plasma sample, (b) Red Cross plasma spiked with 10 ng/ml each of ODV and VEN and 100 ng/ml MAP, (c) subject blank plasma sample spiked with 25 ng/ml MAP, (d) subject plasma sample 1.5 h after venlafaxine adminstration (spiked with 25 ng/ml MAP). Fluorescence parameters: excitation wavelength=276 nm, emission wavelength=598 nm, spectral bandwidth=18 nm, time constant=1.5 s.

Table 1
Sensitivity data and statistics of plasma spiked with 1.00, 5.00 ng/ml VEN, ODV

Theoretical concentration (ng/ml)	VEN		ODV	
	Concentration Found (ng/ml) (mean±S.D.)	C.V. (%)	Concentration Found (ng/ml) (mean±S.D.)	C.V. (%)
1.00	1.16±0.04	3.18	0.46±0.05	10.94
5.00	4.25±0.25	5.79	3.37±0.33	9.88

Table 2
Linear regression parameters of representative standard curves

Parameters	Standard curve 1		Standard curve 2		Standard curve 3	
	VEN	ODV	VEN	ODV	VEN	ODV
Slope	0.0253	0.0140	0.0246	0.0135	0.0251	0.0140
y-Intercept	−0.0110	0.0035	0.0643	0.0364	−0.0371	−0.0235
Number of *x* values	7	7	7	7	7	7
r^2	0.993	0.995	0.999	0.996	0.997	0.995

Table 3
Percent recoveries of VEN and ODV at nominal concentrations 5, 10, 50, 100 ng/ml

Concentration (ng/ml)	% Recovery		
	VEN	ODV	MAP
5	109.08	68.01	
10	90.93	71.26	
50	103.79	66.52	
100	99.33	66.62	53.26
Mean	100.78	68.10	
S.D.	7.68	2.21	
n	4	4	

measurements ($n=25$), at the same nominal concentrations had a C.V.<10% for both VEN and ODV, except for ODV quantitated at 10 ng/ml (C.V.= 13.36%). The accuracy of the method, estimated as the intra-day percentage difference (% diff.) between the mean concentration found and the amount added, had a range of −4.21 to 0.58% for VEN and −7.89 to 2.71%. When the calculations are extended to data collected on five different days ($n=25$), the % difference remained less than 10%.

4. Discussion

This report describes a rapid, accurate and sensitive reversed-phase fluorescence HPLC method to simultaneously ascertain the plasma concentrations of both VEN and ODV. The fluorescence scanning of the pure analytes resulted in four sets of excitation and emission wavelengths: 230, 305 nm; 230, 598 nm; 276, 305 nm and 276, 598 nm (data not shown). The last set of fluorescent wavelengths emerged as the most suitable for this assay (see Fig. 2) having the lowest background noise and little interference from endogenous compounds.

Under the presently prescribed conditions, no loss of VEN occurs during the extraction as approximately 100% recovery over the nominal concentrations 5, 10, 50, 100 ng/ml was observed. To a lesser extent, approximately 70% ODV is recovered. ODV is more polar than VEN, and loss of the free-base ODV during the extraction process may be due to its moderate solubility in the extracting organic solvent (7.5% isoamyl alcohol–hexane). Increasing the amount of the polar isoamyl alcohol would be expected to improve the recovery of the more polar ODV; however, this gain in ODV would have been accomplished at the cost of less VEN recovered, in addition to the possibilities of co-extracting endogenous interfering substances that are not seen under present conditions. The current procedure produces more than sufficient amounts of both VEN and ODV to achieve high sensitivity.

The sensitivity achieved by fluorescence HPLC is normally excellent, and such is the case with our assay. Concentrations as low as 1 ng/ml VEN and 5 ng/ml ODV can be confidently measured (C.V.< 10%, $n=4$, Table 1) providing an advantage over the method using UV detection [7] in which sensitivity of 10 and 7.2 ng/ml for VEN and ODV were obtained. Our method has a high degree of linearity ($r^2>0.99$) between analyte concentrations and peak area ratios; linearity extends to concentrations as

Table 4
Computation of C.V. (%) and % Diff. for intra-day and inter-day (day 1, 2, 3, 4, and 5) measurements of VEN and ODV as the means in assessment of the precision and accuracy of the method

	Amount added (ng/ml)	*n*	Intra-day				Inter-day		
			Amount found (ng/ml) (mean±S.D.)	C.V. (%)	% Diff	*n*	Amount found (ng/ml) (mean±S.D.)	C.V. (%)	% Diff
VEN	10.00	5	9.94±0.46	4.63	−0.65	25	10.56±1.00	9.47	5.60
	50.00	5	47.90±2.57	5.36	−4.21	25	49.38±3.93	7.96	−1.24
	100.00	5	100.58±1.67	1.66	0.58	25	96.43±7.72	8.00	−3.57
ODV	10.00	5	9.21±0.91	9.88	−7.89	25	10.25±1.37	13.36	2.50
	50.00	5	49.49±2.06	4.16	−1.02	25	49.99±3.92	7.84	−0.02
	100.00	5	102.71±2.41	2.35	2.71	25	95.82±8.07	8.42	−4.18

high as 2000 ng/ml, for both VEN and ODV. Another advantage of this method is that it employs a relatively less hydrophobic C_4 column resulting in short elution time of less than 20 min per analysis, saving both time and mobile phase. Also mild operating conditions using neutral buffer (40 m*M* sodium phosphate, pH 6.8) are likely to prolong column life.

The differences of less than 10% for both the intra-day and inter-day data reflect the accuracy of this method. The observation of C.V.$<$10% for intra-day, and $<$15% for inter-day measurements also indicate a high degree of precision. This finding attests to reliability in delivering accurate and precise determination of VEN and ODV in human plasma. The excellent specifications together with the rapidity of the procedures suggest that this method should be considered when a clinical need arises for determining plasma VEN and ODV levels. In fact, in our clinical study investigating the effect of VEN on imipramine metabolism (data not shown), mean steady state plasma levels of VEN and ODV, measured at 48th hour, for six subjects (thrice daily oral dosage of 50 mg VEN) were found to be 185.79±103.73 and 462.82±247.74 ng/ml, respectively.

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