Journal of Pharmaceutical Analysis 2013;3(6):466–471

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

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ORIGINAL ARTICLE

Rapid sensitive validated UPLC–MS method for determination of venlafaxine and its metabolite in rat plasma: Application to pharmacokinetic study

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Received 5 October 2012; accepted 13 May 2013
Available online 3 June 2013

KEYWORDS

Venlafaxine;
O-desmethylvenlafaxine;
Metabolite;
UPLC–MS/ES

Abstract A new ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–MS/ESI) method for simultaneous determination of venlafaxine (VEN) and its metabolite O-desmethyl-venlafaxine (ODV) in rat plasma has been developed and validated using Venlafaxine d6 as the internal standard. The compounds and internal standard were extracted from plasma by solid phase extraction. The UPLC separation of the analytes was performed on ACQUITY UPLC® BEH Shield RP18 (1.7 μm, 100 mm × 2.1 mm) column, using isocratic elution with mobile phase constituted of water (containing 2 mM ammonium acetate): acetonitrile (20:80, v/v) at a flow rate of 0.3 mL/min. All of the analytes were eluted within 1.5 min. The compounds were ionized in the electrospray ionization (ESI) ion source of the mass spectrometer, operating in multiple reaction monitoring (MRM) and positive ion mode. The precursor to product ion transitions monitored for VEN, ODV and Venlafaxine d6 were *m/z* 278.3→121.08, 264.2→107.1 and 284.4→121.0, respectively. The developed and validated method was used for the pharmacokinetic study of VEN in rats.

1. Introduction

Venlafaxine (VEN), chemical structure 1-[2-(dimethylamino)-1-(4-methoxy-phenyl)ethyl] cyclohexanol hydrochloride, is a new antidepressant, which selectively inhibits re-uptake of norepinephrine and serotonin, and slightly inhibits re-uptake of dopamine, without significant affinity for muscarinic, histaminergic or adrenergic receptors [1]. In humans VEN is metabolized into two minor metabolites, N-desmethylvenlafaxine (NDV), N,O-didesmethylvenlafaxine (DDV), and the major active metabolite, O-desmethylvenlafaxine (ODV) which presents an activity profile

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Peer review under responsibility of Xi'an Jiaotong University.

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http://dx.doi.org/10.1016/j.jpha.2013.05.002

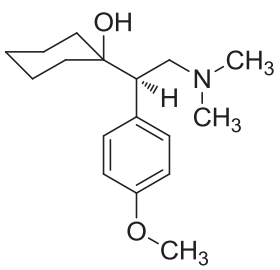
similar to that of VEN [2–6]. The biotransformation of VEN into ODV is controlled by CYP2D6, CYP2C19 and CYP2C9, with estimated contributions of 89%, 10% and 1%, respectively [2–6]. There are several published methods for the determination of both VEN and ODV in the biological matrix including CE, GC and HPLC [7–9]. The reported HPLC method, using HPLC coupled with mass spectrometry or coulometric detection, has high sensitivity by using the solid-phase exaction (SPE) [9,10], while others using fluorescence or UV detection have low sensitivity or long analysis time (>10 min) [11–14]. The reported ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–MS/ESI) [15] has a disadvantage of using diethylether which is toxic and flammable and uses high plasma aliquot volume for sample preparation.

The aim of the present study is to establish a simple, rapid and accurate UPLC–MS/ESI method for the measurement of VEN and ODV in pharmacokinetic samples.

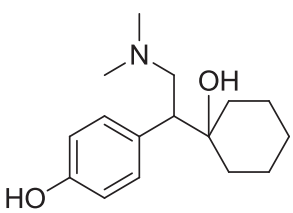
2. Experimental

2.1. Equipments and reagents

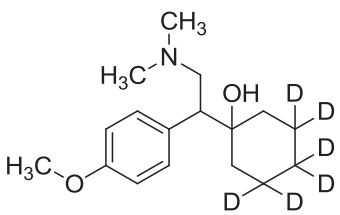
A system of UPLC with a Micro mass ZQ mass spectrometer (Wythenshawe, Manchester, UK) with mass-selective detector equipped with an electrospray ionization (ESI) ion source was used. Lenovo Workstation and MassLynx 4.1 software were utilized. Venlafaxine, O-desmethylvenlafaxine and Venlafaxine d6 were obtained from Sigma Aldrich. The structures of these compounds are presented in Fig. 1.



Venlafaxine (VEN)



O-Desmethylvenlafaxine (ODV)



Venlafaxine d6

Fig. 1 Chemical structure of each compound: Venlafaxine (VEN), O-desmethylvenlafaxine (ODV) and Venlafaxine d6.

MS grade reagents (methanol, acetontritile) and diethylether were purchased from Merck, India. Triple distilled water was prepared by a laboratory purification system.

2.2. Standard and working solutions

The primary stock solutions of VEN (2 mg/mL), ODV (2 mg/mL), and Venlafaxine d6 (1 mg/mL) were prepared by dissolving appropriate amounts of pure substance in methanol. Working solutions were obtained by diluting the stock solutions with methanol. All the standard solutions were stored at 4° C. Routine daily calibration curves were prepared in drug-free plasma. Appropriate volumes of working solution and drug-free rat plasma were added to each test tube. Final concentrations were 10, 25, 50, 100, 250, 500, 1000 and 2000 ng/mL for VEN and ODV. Quality control (QC) samples, which were run in each assay, were prepared in the same way.

2.3. Chromatographic conditions

Chromatographic separation of the analytes was performed on ACQUITY UPLC® BEH Shield RP18 (1.7 μm, 100 mm × 2.1 mm) column with column temperature 35 °C. The mobile phase consisted of water (containing 2 mM ammonium acetate) and acetonitrile (20:80, v/v) at a flow rate of 0.3 mL/min.

2.4. Mass spectrometric conditions

For optimization of MS parameters 500 ng/mL of each analyte was prepared in methanol. Argon was used as collision gas and nitrogen as nebulizer gas. The compounds were ionized in the positive electrospray ionization ion source (ESI+) of the mass-spectrometer. The product ion spectrum (MS–MS) was generated under optimized conditions to identify the prominent product ions of the analytes. Multiple reaction monitoring (MRM) mode was used for quantitation of each analyte depicted in Table 1.

2.5. Sample preparation

Sample preparation involved a simple solid phase extraction with OASIS HLB (Hydrophilic lipophilic balance) cartridges. Twenty microliters internal standard working solution (Venlafaxine d6, 50 μg/mL) was added to 300 μL aliquots of blank/spiked plasma and vortexed. The cartridges were conditioned with methanol (1 mL) and equilibrated with de-ionized water (1 mL). The prepared samples were then loaded onto cartridges and the

Table 1 Optimized MS/MS parameters.

Analyte	Parent ion	Product ion	Cone voltage (V)	Collision voltage (V)	Capillary voltage (kV)
VEN	278.3	121.1	27	24	3.50
ODV	264.2	107.1	26	24	3.50
Venlafaxine d6	284.4	121.0	12	42	3.50

cartridges were washed with 5% methanol (1 mL) and finally eluted with methanol (1 mL). The eluates were evaporated to dryness at 40° C under N_2 gas. Residues were then reconstituted in 200 μL of mobile phase. Seven microliters of the solution was injected for analysis through the auto-injector.

2.6. Method validation

The method was validated in terms of linearity, specificity and selectivity, lower limit of detection (LOD) and lower limit of quantification (LLOQ), recovery, matrix effect, accuracy, precision and stability. These parameters determined were carried out in six replicates at low, medium and high concentration levels.

2.6.1. Linearity

Biological samples were quantified using the ratio of peak area of VEN, ODV to that of internal standard. The calibration curves were established through a linear least-squares regression with a weighing factor of $1/C^2$, where C is the concentration of the calibration standards. Coefficients of correlations (r) were required to be 0.99 or better. Concentration in the quality control was quantified from the regression equation.

2.6.2. Specificity and selectivity

Interference in biological matrix arises from a number of endogenous sources (analyte, metabolite, degradation products occurring in biological fluids) and exogenous sources. The selectivity of this method was investigated by analyzing six individual blank plasma samples. Each blank sample was tested for interferences using the proposed extraction procedure and LC–MS/MS conditions.

2.6.3. LOD and LLOQ

The LODs for VEN and ODV were defined as the drug concentration in the plasma after the sample preparation method that corresponds to three times the baseline noise $S/N \geq 3$. The LLOQ was defined as the concentration of the sample that can be quantified with $<20\%$ deviation ($S/N \geq 10$).

2.6.4. Recovery

The percentage recovery of VEN, ODV and internal standard was determined by measuring the mean peak area response of six replicates of extracted QC samples at low, middle and high levels against the mean peak area response of six replicates of aqueous (unextracted) QC samples at concentrations representing 100% extraction of QC samples at low, middle and high concentrations.

2.6.5. Matrix effect

Matrix effect is due to co-elution of some endogenous components present in biological samples. These components may not give a signal in MRM of the starget analyte, but can certainly decrease or increase the response of the analyte dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS method. To evaluate the matrix effect, chromatographic peak areas of VEN, ODV from the spike-after preparation samples were compared to those of the neat standards at the QC concentrations.

2.6.6. Accuracy and precision

The precision and accuracy of the method were assessed by intra- and inter-day validation. The intra- and inter-day accuracy and precision were determined by determining the concentrations of VEN and ODV in plasma in six replicates of QC samples for three separate batches. Precision was expressed as % CV.

2.6.7. Stability

Stability experiments were performed to evaluate stabilities of the analytes in plasma samples under different storage and processing conditions. All stability exercises were assessed by analyzing six replicates of stability samples at low (LQC) and high quality control (HQC) levels against freshly spiked calibration standards and comparing with freshly spiked six replicates of samples at LQC and HQC levels. Freeze–thaw stability of spiked plasma samples was determined after three freeze–thaw cycles. Each cycle consisted of removing the samples from the freezer, thawing them unassisted to room temperature and keeping at room temperature for 2 h and re-freezing at −50° C. Bench top stability evaluation involved analysis of stability samples, which had been kept at room temperature for a period of 7.5 h. Post preparative stability involved analysis of stability samples, which were kept in a refrigerator for 24 h. Stock solution stability was determined by storing stability stock solutions in a refrigerator between 1 and 10 °C. After 10 days fresh stocks were prepared. A dilution with the same concentration from each of the stability and fresh stocks was prepared and six replicate injections were given. Short term stability of stock dilution was determined by keeping stability stock dilution at room temperature for 9 h and comparing it with fresh stock dilution made from the same stock.

2.7. Pharmacokinetics of venlafaxine in rats

Seven healthy male Wistar strain rats weighing 180–220 g were used in the study. An oral dose of venlafaxine 30 mg/kg was given to each rat. The study was done for 24 h and the blood samples were drawn at 0.25, 0.5, 1, 2, 3, 6, 8, 12, 16 and 24 h after administration of drug. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and centrifuged at 12,000 rpm for 30 min at 5° C. The resulting plasma samples were stored at −20 °C until analysis. The plasma samples were extracted by HLB cartridges as described above and analyzed for VEN and ODV. Different pharmacokinetic parameters were determined using WinNonlin software.

3. Results and discussion

3.1. UPLC–MS/ESI

The column temperature was 35 °C in order to reduce pressure of column and improve resolution. Seven microliters of reconstituted solution was injected for analysis in order to improve the sensitivity. The UPLC–MS/ESI in the MRM mode provided a highly selective method for the determination of VEN and ODV. The retention times of ODV and VEN were approximately 0.83 and 0.93 min, respectively.

Compared with the published methods (the chromatographic run was as long as 10–20 min) [16–18], the run time of this method was shorter, i.e., complete elution was obtained in less than 1.5 min. The chromatograms of VEN, ODV and Venlafaxine d6 are depicted in Fig. 2.

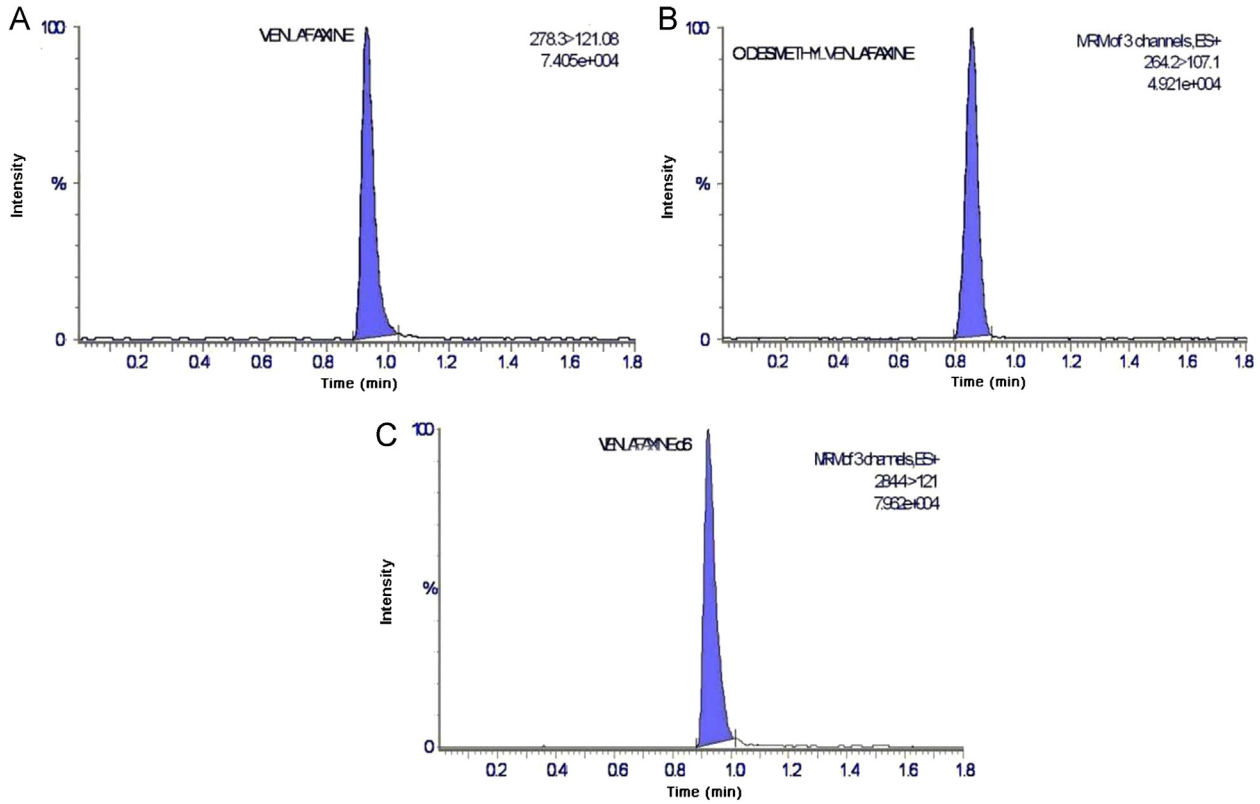


Fig. 2 Representative chromatograms of (A) VEN, (B) ODV and (C) Venlafaxine d6.

Table 2 Absolute recoveries of VEN and ODV from rat plasma.

Concentration (ng/mL)	Absolute recovery (% mean ± SD, $n=6$)	
	VEN	ODV
20 (LQC)	78.4 ± 2.6	76.4 ± 2.4
400 (MQC)	82.6 ± 6.7	80.6 ± 5.8
1600 (HQC)	90.6 ± 2.2	88.6 ± 2.3

3.2. *Assay characteristics for method validation*

3.2.1. *Linearity and calibration standards*

The concentration ranges were 10–2000 ng/mL for VEN and ODV. The area ratio of each analyte to internal standard was well related to the concentration. The data were based on three replicates of an eight-point calibration curve and the linear relationships were described by the following equations:

$$\text{VEN}: \; y = 0.0051x + 0.00084; \quad R^2 = 0.993$$

$$\text{ODV}: \; y = 0.0020x + 0.01219; \quad R^2 = 0.992$$

3.2.2. *Specificity and selectivity*

Chromatograms of six batches of control drug-free plasma contained no co-eluting peak >20% of analyte area at LLOQ level and no co-eluting peaks >5% of the area of internal standard. There was no cross interference between analytes after subjecting individual analytes. Representative chromatograms of extracted VEN and ODV are shown in Fig. 2. The retention times of both analytes and IS showed less variability, with a relative standard deviation well within the acceptance limit of 5%.

3.2.3. *LOD and LLOQ*

The LOD demonstrated that both VEN and ODV gave an *S/N* ratio ≥3 of 2.66 ng/mL and 2.78 ng/mL respectively. The LLOQ was 7.98 ng/mL for VEN and 8.34 ng/mL for ODV.

3.2.4. *Recovery*

The extraction recovery was determined at three concentrations of 20, 400 and 1600 ng/mL as described in Table 2.

The percentage recovery of Venlafaxine d6 acid was determined by measuring the mean peak area response of Venlafaxine d6 acid in extracted QC samples (MQC) against the mean peak area response of aqueous (unextracted) internal standards solution at a concentration representing 100% extraction of internal standards in MQC samples. The recovery of Venlafaxine d6 was 88.8%.

3.2.5. *Accuracy and precision*

The accuracy of the assay was defined as the absolute value of the calculated mean concentration of the QC sample to their respective nominal values, expressed as percentage. The precision of the assay was measured by the percent coefficient of variation at concentrations of LQC. MQC and HQC levels. The results are presented in Table 3.

3.2.6. *Stability*

Analytes were stable in plasma for three freeze–thaw cycles. Bench top stability for analytes was established for 7.5 h. Post preparative stability indicated samples were stable for 24 h after processing. The results of the stability exercises mentioned above are given in Table 4. The stock solutions of VEN and ODV were found to be stable for 10 days when stored in the refrigerator (1–10° C). The percentage stability values of the stock solutions for VEN and ODV were 106.3% and 94.2% respectively.

Short-term stability for VEN and ODV stock dilutions was established for 9 h at room temperature. The stability values of the stock dilutions for VEN and ODV were 98.1% and 97.7% respectively. The precision was 0.8% for stability and 0.7% for fresh dilutions for VEN. The precision was 1.2% for stability and 0.8% for fresh dilutions for ODV.

3.3. *Pharmacokinetic application*

This validated UPLC–MS/ESI method was successfully applied to a pharmacokinetic study, for simultaneous determination of VEN and its active metabolite following oral administration of 30 mg/kg venlafaxine hydrochloride in rats. The mean plasma concentration–time curve of VEN and ODV in single dose study is shown in Fig. 3. The pharmacokinetic parameters of VEN and ODV are given in Table 5. By observing the pharmacokinetic parameters, the elimination half lives of VEN and ODV were comparable; plasma clearance and volume of distribution of VEN and ODV are high and similar. Based on T_{max} value, VEN gets absorbed and metabolized into ODV rapidly. Based on C_{max} and AUC values it is evident that exposure to ODV was less than VEN when compared to humans; this might be due to conversion of more VEN into cis-1,4-dihydroxycyclohexyl venlafaxine (major metabolite in rats) or more formed ODV is metabolized further. Since metabolism of VEN into ODV is dose dependent (saturation kinetics), a higher dose (30 mg/kg) of VEN also leads to less exposure of ODV when compared to VEN.

Table 3 Accuracy (% basis) and precision (%RSD) of analytes.

Analyte	Concentration (ng/mL)	Accuracy (% basis)		Precision (% RSD)	
		Intra-day	Inter-day	Intra-day	Inter-day
VEN	20 (LQC)	2.52	4.26	3.26	4.74
	400 (MQC)	5.25	5.20	4.52	5.25
	1600 (HQC)	3.55	3.80	2.20	3.86
ODV	20 (LQC)	5.06	6.28	5.25	5.10
	400 (MQC)	3.54	4.75	4.25	2.06
	1600 (HQC)	4.25	3.86	6.25	7.25

4. Conclusion

This method had a suitable limit of quantification along with the very short retention time of 1.5 min which can be useful in

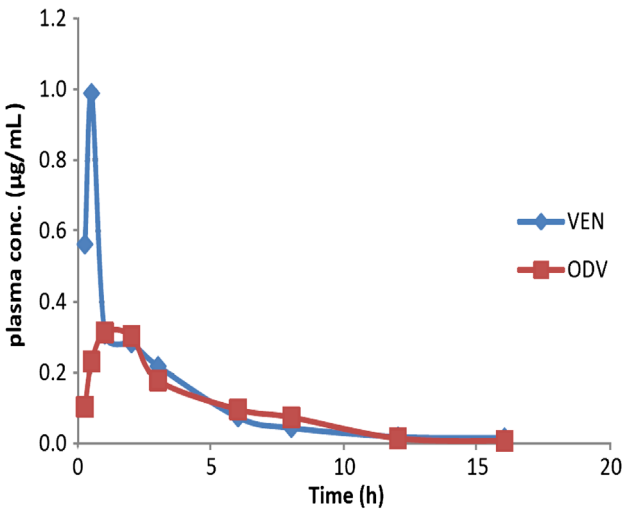


Fig. 3 Plasma concentration–time profile of VEN and ODV after oral administration of 30 mg/kg of venlafaxine hydrochloride in rats.

Table 5 Pharmacokinetic parameters of VEN and ODV.

Parameters	VEN	ODV
T_{max} (h)	0.5	1.0
C_{max} (µg/mL)	1.48	0.47
AUC (µg h/mL)	2.92	2.33
V_d (L/kg)	185.58	–
Cl (L/h/kg)	41.03	–
$t_{1/2}$ (h)	3.13	2.59

Table 4 Stability of VEN and ODV in rat plasma.

Parameters	Concentration (ng/mL)	VEN		ODV	
		CV (%)	% Nominal	CV (%)	% Nominal
Bench top stability (for 7.50 h)	20 (LQC)	1.2	103.2	1.5	103.2
	1600 (HQC)	1.5	101.5	1.1	98.5
Freeze–thaw stability (for three cycles)	20 (LQC)	1.1	102.5	1.3	105.2
	1600 (HQC)	1.6	105.4	0.8	101.5
Post-processing refrigerator stability (for 24 h)	20 (LQC)	1.5	102.9	0.9	106.2
	1600 (HQC)	1.8	99.2	1.6	101.5

analyzing many samples in a single day which is the most advantageous aspect in comparison to other analysis techniques available in published literature. This method employs a very simple cost effective sample preparation technique as well as very less injection volume (7 μL) resulting in high throughput bioanalysis technique. The method had advantage over problems of poor chromatography, questionable uncharacterized peak, tedious extraction steps, and high injection load. Our research thus confidently demonstrates for the first time a simple, robust and high throughput method requiring less than 1.5 min to run each sample. Thus, as the pace of drug development quickens, we cannot ignore the potential for our method application nor the time saving which can present thus encouraging clinical and exploratory studies for the best possible antidepressant agents.

Animal rights

The institutional and international guide for the care and use of laboratory animals was followed. See the experimental part for details.

Acknowledgment

The authors are thankful to University Grant Commission, New Delhi for extending their financial support.

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