Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 829 (2005) 75–81
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for simultaneous determination of venlafaxine and its active metabolite *O*-desmethyl venlafaxine in human plasma

Jignesh Bhatt[a,b,*], Arvind Jangid[a], Gantala Venkatesh[a], Gunta Subbaiah[a], Sadhana Singh[b]

[a] *Bioanalytical Department, Torrent Research Centre, Near Indira bridge, Village Bhat, Gandhinagar 382428, Gujarat, India*
[b] *Department of Chemistry, B.N.P.G. College, Mohanlal Sukhadia University, Udaipur, Rajasthan, India*

Received 19 July 2005; accepted 23 September 2005
Available online 24 October 2005

Abstract

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method has been developed and validated for simultaneous quantification of venlafaxine (VEN) and *O*-desmethyl venlafaxine (ODV) in human plasma. The analytes were extracted from human plasma by using solid-phase extraction (SPE) technique. Escitalopram (ESC) was used as the internal standard. A Betasil C18 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The mass transition ion-pair has been followed as *m*/*z* 278.27 → 121.11 for VEN, *m*/*z* 264.28 → 107.10 for ODV and *m*/*z* 325.00 → 262.00 for ESC. The method involves a solid phase extraction from plasma, simple isocratic chromatography conditions and mass spectrometric detection that enables detection at nanogram levels. The proposed method has been validated with linear range of 3–300 ng/ml for VEN and 6–600 ng/ml for ODV. The intrarun and interrun precision and accuracy values are within 10%. The overall recoveries for VEN and ODV were 95.9 and 81.7%, respectively. Total elution time as low as 3 min only.

Keywords: Venlafaxine; *O*-Desmethyl venlafaxine; LC–MS–MS; Human plasma

1. Introduction

Venlafaxine (VEN), 1-2-(dimethylamino)-1-(4-methoxyphenyl) ethyl-cyclohexanol hydrochloride (Fig. 1), is a new phenethylamine bicyclic antidepressant, which has a neuropharmacologic profile distinct from that of existing antidepressants including tricyclic compounds [1,2]. It imparts antidepressant effects by inhibiting the neuronal uptake of norepinephrine, serotonin and dopamine and lacks the adverse side effects profile of tricyclic antidepressants [3–6].

VEN is well absorbed in humans and undergoes extensive metabolism in the liver and has several metabolites, one of which is biologically active. VEN is extensively metabolized to *O*-desmethyl venlafaxine (ODV) (Fig. 1), a major metabolite with an activity profile similar to that of VEN [5,7]. To establish pharmacokinetic parameters it is therefore important to monitor plasma concentration of both VEN and ODV.

Different methods have been reported in the literature for monitoring plasma levels of VEN and ODV [8–21]. The techniques used in these methods include capillary electrophoresis mass spectrometric detection [8,9], Capillary electrophoresis [10–15], HPLC) with fluorimetric detection [16,17], HPLC with coulometric detection [18] and HPLC with UV detection [19–21]. The analytical method reported requires laborious extraction procedure like liquid–liquid extraction or solid-phase extraction (SPE) involving drying and reconstitution, long run time and high quantification limit, therefore it was necessary to develop a simple, specific, rapid and sensitive analytical method for the quantification of the VEN and its active metabolite ODV.

This paper describes development and validation of a simple, specific, rapid and sensitive Liquid chromatography–tandem mass spectrometry method for the determination of VEN and

* Corresponding author. Tel.: +91 79 23969100; fax: +91 79 23969135.
E-mail address: jigu_baroda@rediffmail.com (J. Bhatt).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.09.034

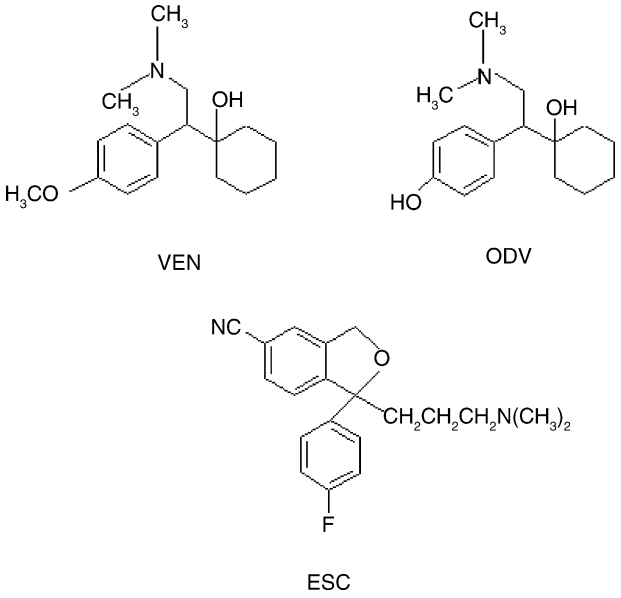

Fig. 1. Chemical Structure of VEN, ODV and ESC.

ODV in human plasma with a limit of quantification (LOQ) of 3 and 6 ng/ml for VEN and ODV, respectively with a run time of 3.0 min using Escitalopram (ESC) (Fig. 1) as internal standard.

2. Experimental

2.1. Chemicals and reagents

The reference standards of VEN (purity: 99.67%), ODV (purity: 97.96%) and ESC (purity: 98.44%) were obtained from Torrent Research Centre (Ahmedabad, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (India) Pvt. Ltd. (Bangalore, India). Gradient grade methanol and acetonitrile were purchased from E. Merck Ltd. (Mumbai, India). Suprapure ammonium acetate and formic acid were purchased from Merck (Germany). General reagent grade sodium hydroxide was purchased from Merck (India). Drug free (blank) heparinized human plasma was obtained from Green cross laboratory (Ahmedabad, India) and were stored at −20 °C prior to use.

2.2. Calibration curves

The Stock solutions of VEN, ODV and internal ESC were prepared in methanol at free base concentration of 1000 μg/mL. Secondary and working standard solutions were prepared from stock solutions by dilution with water:methanol (50:50, v/v). This diluted working standard solutions were used to prepare the calibration curve and quality control samples.

Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of VEN, ODV and internal standard ESC. A nine point standard curve of VEN and ODV was prepared by spiking the blank plasma with appropriate amount of VEN and ODV. The calibration curve ranged from 3.0 to 300 ng/ml and 6.0 to 600 ng/ml for VEN and ODV, respectively. Quality control samples were prepared at three concentration levels of 9.0, 75.0 and 225.0 ng/ml for VEN and 18.0, 150.0 and 450.0 ng/ml for ODV in a manner similar to the standard from the stock solution.

2.3. Sample preparation

A 0.5 ml aliquot of human plasma sample was mixed with 25 μl of internal standard working solution (1.5 μg/ml of ESC) and 0.5 ml of 0.005 M Sodium hydroxide. The sample mixture was loaded into an oasis HLB extraction cartridge that was preconditioned with 1 ml methanol followed by 2 ml water. The extraction cartridge was washed with 2 ml of water followed by 1 ml 5% methanol. VEN, ODV and ESC were eluted with 1 ml of acetonitrile, 5 μl of the eluent was injected into the LC–MS–MS system.

2.4. Instrumentation

Chromatographic separation was carried out on Surveyor HPLC with Betasil C18 column (3 μm, 100 mm × 3.0 mm) purchased from Thermo electron corporation, UK. A mobile phase consisting of acetonitrile and ammonium acetate (pH 3.5, 5 mM) (75:25, v/v) was delivered with a flow rate of 0.3 ml/min. The total run time for each sample analysis was 3.0 min. Mass spectra were obtained using a TSQ Quantum mass spectrometer (Thermofinnigan Ltd. UK) equipped with electrospray ionisation (ESI) source. The mass spectrometer was operated in the selected reaction-monitoring (SRM) mode. Sample introduction and ionization was electrospray ionization in the positive ion mode. The spray voltage and capillary temperature were 3400 V and 350 °C, respectively. Argon gas was used as collision gas. The mass transition ion-pair was selected as m/z 278.27 → 121.11 for VEN, m/z 264.28 → 107.10 for ODV (Fig. 2) and m/z 325.00 → 262.00 for ESC. The data acquisition was ascertained by Xcalibur 1.3 software. For quantification the peak area ratios of the target ions of the drugs to those of the internal standard were compared with weighted ($1/c$) least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

2.5. Validation

The method has been validated for selectivity, sensitivity, linearity, precision, accuracy, recovery and stability. Selectivity was performed by analyzing the blank plasma samples from different sources (or donors) to test for interference at the retention time of VEN, ODV and internal standard ESC. The intrarun and interrun accuracy was determined by replicate analysis of quality control samples and at LOQ that were extracted from the sample batch.

Accuracy is defined as the percent relative error (%R.E.) and was calculated using the formula %R.E. = $(E - T)(100/T)$ where E is the experimentally determined concentration and T is the-

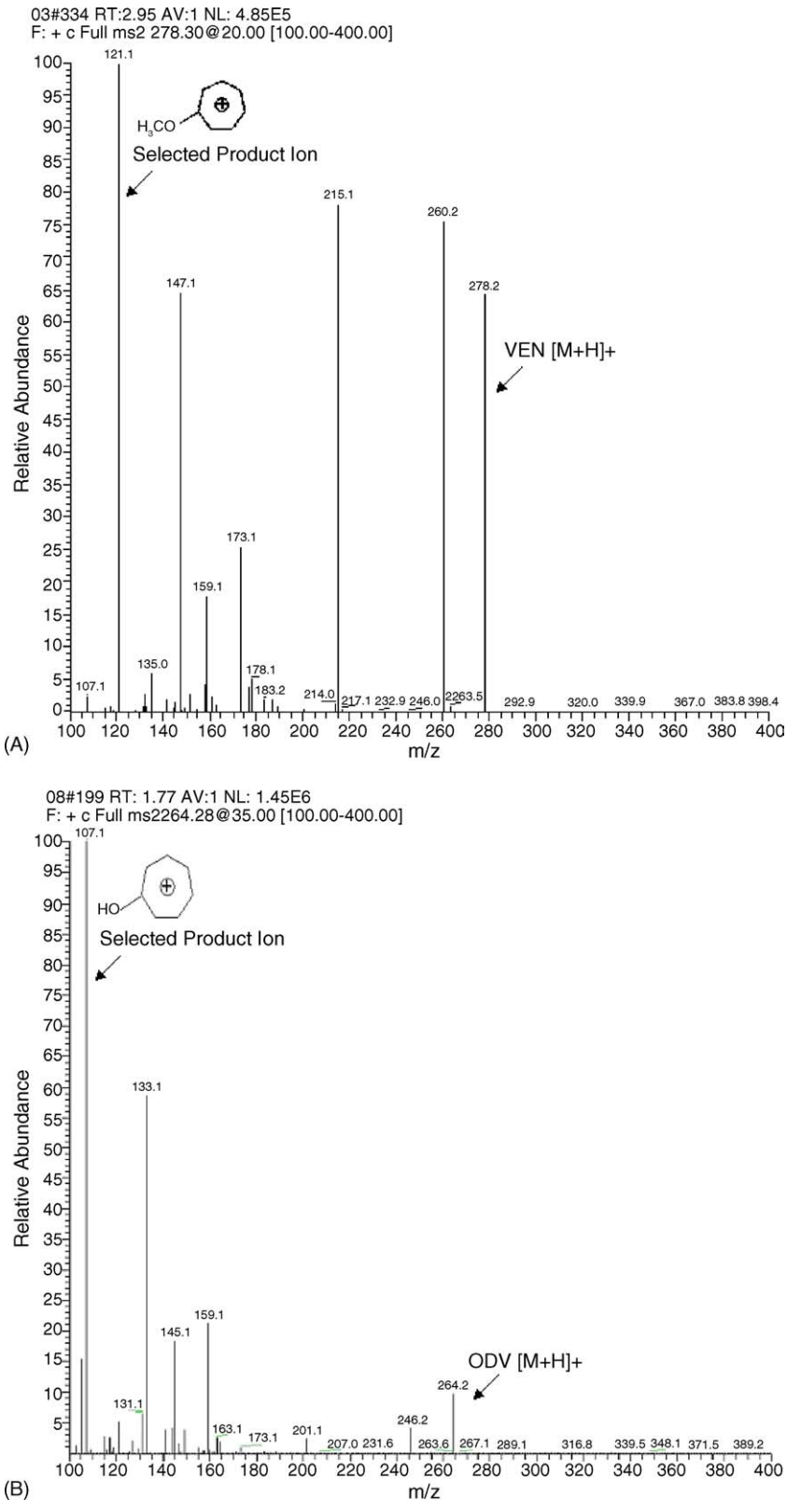

Fig. 2. Electrospray product ion mass spectra of the precursor ions of: VEN(A) and ODV (B).

oretical concentration. Assay precision was calculated by using the formula %R.S.D. = (S.D./M) × 100 where M is the mean of the experimentally determined concentrations and S.D. is the standard deviation of M.

The extraction efficiencies of VEN, ODV and ESC were determined by analysis of five replicates at low, medium and high quality control concentrations for VEN, ODV and at one concentration for the internal standard, ESC. The percent recovery

was evaluated by comparing the peak area of extracted analytes to the peak area of non-extracted standards.

As a part of the method validation, stability was evaluated. Analytes were considered stable if the %R.E. of the mean test responses were within 15% of appropriate controls. Analytes were tested using the quality control samples whenever appropriate. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 5 °C for 24 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. Five aliquots of each low and high concentration were used for the freeze–thaw stability evaluation. The stability of spiked human plasma stored at −70 °C (long-term stability) was evaluated by analyzing low, medium and high quality control samples that were stored at −70 °C for 98 days together with freshly spiked calibration standard and quality control samples.

3. Results and discussion

3.1. Method development

The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the simultaneous extraction and quantification of VEN and ODV, suitable for determine the pharmacokinetics of these compound in clinical studies. To achieve the goal, during method development different options was evaluated to optimize sample extraction, detection parameters and chromatography. Since the VEN and ODV was found to exists as a unionized form in the basic pH, in this assay, the plasma samples were treated with 0.005 M sodium hydroxide and loaded on to the HLB SPE cartridge. In the state of non-ionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. Electrospray ionization and atmospheric pressure chemical ionization (APCI) was evaluated to get better response of analytes. It was found that the best signal was achieved with ESI positive ion mode. APCI did not show any advantages over ESI. Analytes shown increased signal with reducing the pH of diluents.

Further optimization in chromatography conditions increased signal of analytes. A mobile phase containing 5 mM ammonium acetate (pH 3.5) buffer in combination with acetonitrile resulted in improved signal. Use of short Betasil C18 (100 mm × 3 mm i.d., 3 μ) column resulted in reduced flow rate and reduce run time as low a 3.0 min. The column oven temperature was optimized to 40 °C in order to get symmetry of analyte peaks. The resulted signal with optimized chromatography and detection parameters enabled to eliminate the laborious extraction steps of evaporation of eluent and reconstitution involved in generic SPE methods without compromising the sensitivity, which further resulted in reduced processing and analysis time.

3.2. Selectivity

Representative chromatogram obtained from blank plasma, plasma spiked with LOQ standard and extracted subject sample is presented in Figs. 3 and 4, respectively. No interfering peak of endogenous compounds observed at the retention time of analytes in blank human plasma of six different lots, lipemic and heamolysed plasma. Utilization of predominant product ions for each compound enhanced mass spectrometric selectivity. The predominant product ions of *m*/*z* 121.11, 107.10 and 262.00 were specific for VEN, ODV and ESC.

3.3. Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 3.0–300.0 ng/ml for VEN and 6.0–600.0 ng/ml for ODV. The calibration curves appeared linear and were well described by least squares lines. A weighing factor of 1/concentration was chosen to achieve homogeneity of variance. The correlation coefficients were ≥0.9992 for VEN and ≥0.9989 for ODV. The mean (±S.D.) slope of calibration curves for VEN and ODV was 0.013408 (±0.001102) and 0.008478(±0.000527), respectively. The mean intercept of calibration curves for VEN and ODV was 0.00648 (±0.00170) and 0.00842 (±0.002269), respectively.

3.4. Sensitivity (lower limit of quantification)

The LOQ for VEN and ODV is 3.0 and 6.0 ng/ml, respectively. These data are tabulated in Table 1 for VEN and ODV. The intrarun precision of the LOQ plasma samples containing VEN and ODV is 7.67 and 3.37%, respectively. The intrarun accuracy of the LOQ plasma samples containing VEN and ODV is 0.16 and 7.37%, respectively.

3.5. Precision and Accuracy

The intrarun precision was ≤7.67% for VEN and ≤3.37% for ODV, respectively (Table 1). The intrarun accuracy was ≤2.40 and ≤7.37% for VEN and ODV, respectively (Table 1). The interrun precision and accuracy were determined by pooling all individual assay results of replicate ($n = 5$) quality control

Table 1
Intrarun precision and accuracy ($n = 5$) of VEN and ODV in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%R.E.
VEN	3.000	3.005	7.67	0.16
	9.000	8.784	3.60	−2.40
	75.000	76.598	1.63	2.13
	225.000	226.332	1.80	0.59
ODV	6.000	5.558	3.37	−7.37
	18.000	17.785	0.69	−1.20
	150.000	157.057	2.01	4.70
	450.000	458.041	2.27	1.79

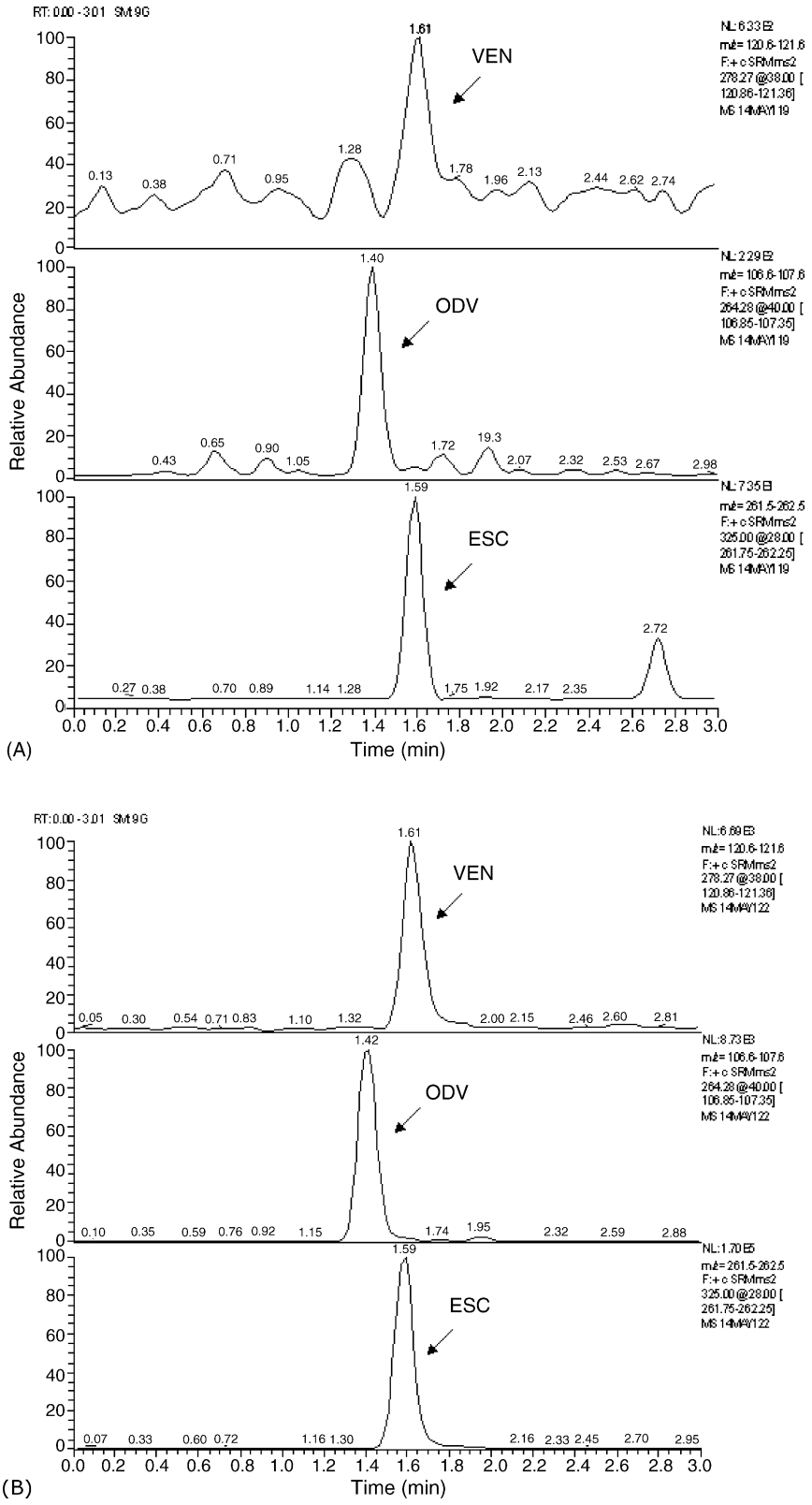

Fig. 3. Representative chromatograms of extracted blank plasma (A) and extracted plasma LOQ sample (B).

over the three separate batch runs. The interrun precision was ≤6.31% for VEN and ≤6.82% for ODV, respectively (Table 2). The interrun accuracy was 3.96 and 7.56% for VEN and ODV, respectively (Table 2).

3.6. Recovery

Five replicates at low, medium and high quality control concentration for the VEN and ODV were prepared for recovery

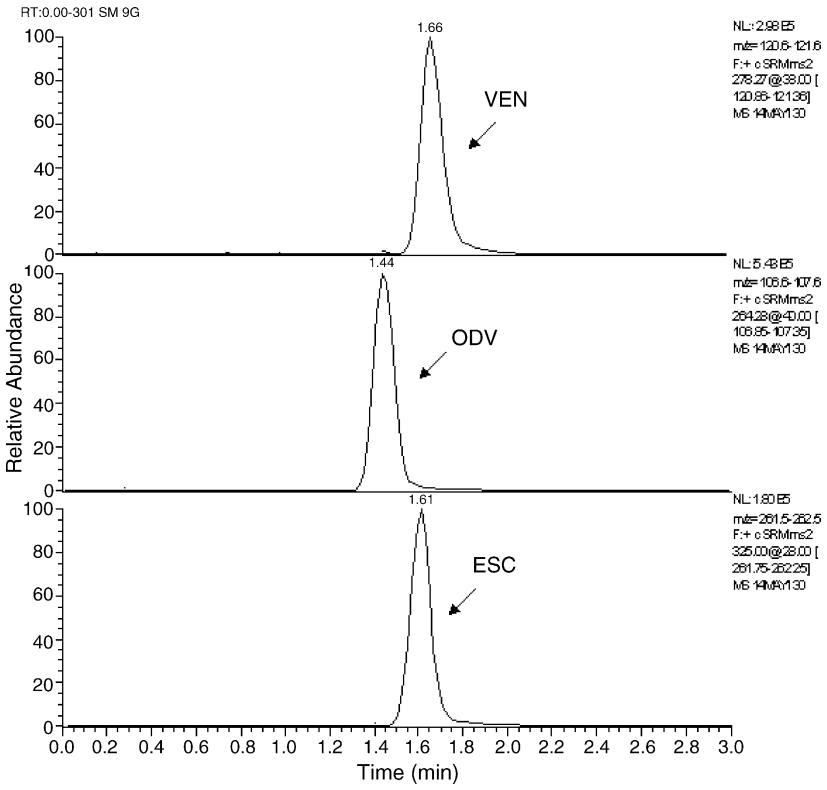

Fig. 4. Representative chromatograms of extracted subject sample.

determination. The Mean recovery for VEN and ODV were 95.86 and 81.67% with precision of 2.11 and 0.96%, respectively. The mean recovery for ESC was 91.45%.

3.7. Stability

Bench top and process stabilities for VEN and ODV were investigated at LQC and HQC levels. The results revealed that VEN and ODV were stable in plasma for at least 6 h at room temperature and 24 h at auto sampler (Tables 3 and 4). It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with VEN and ODV at LQC and HQC level did not effect the stability of VEN and ODV (Tables 3 and 4). Long-term stability for VEN and ODV in plasma at −70 °C was performed at LQC, MQC and HQC level. The results revealed that VEN and ODV were stable for at least 98 days (Tables 3 and 4).

Table 2
Interrun precision and accuracy ($n = 3$) of VEN and ODV in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%R.E.
VEN	3.000	2.881	6.31	−3.96
	9.000	8.928	3.14	−0.80
	75.000	76.722	2.98	2.30
	225.000	225.957	2.50	0.43
ODV	6.000	5.547	6.82	−7.56
	18.000	17.550	4.50	−2.50
	150.000	152.595	3.26	1.73
	450.000	443.766	3.94	−1.39

3.8. Application of method

The proposed method was applied to the determination of VEN and ODV in plasma samples from an on going project for the development of a sustained release formulation. Plasma samples were periodically collected upto 96 h after oral single dose administration of 150 mg sustained release tablet to 24 healthy

Table 3
Stability results for VEN ($n = 5$)

Stability	Spiked concentration (ng/ml)	Mean (±S.D.) obtained concentration (ng/ml)	%R.E.
Process[a]	9.000	8.993 (±0.236)	−0.08
	225.000	220.988 (±3.159)	−1.78
Bench top[b]	9.000	8.925 (±0.335)	−0.83
	225.000	222.078 (±2.380)	−1.30
Freeze and thaw[c]	9.000	8.927 (±0.139)	−0.81
	225.000	217.445 (±3.225)	−3.36
Long-term[d]	9.000	8.555 (±0.225)	−4.94
	75.000	71.199 (±1.756)	−5.07
	225.000	207.504 (±2.858)	−7.78

[a] After 24 h in autosampler at 5 °C.
[b] After 6 h at room temperature.
[c] After 3 freeze and thaw cycles at −70 °C.
[d] At −70 °C for 98 days.

Table 4
Stability results for ODV ($n = 5$)

Stability	Spiked concentration (ng/ml)	Mean (±S.D.) obtained concentration (ng/ml)	%R.E.
Process[a]	18.000	17.934 (±0.245)	−0.37
	450.000	445.604 (±6.644)	−0.98
Bench top[b]	18.000	18.283 (±0.567)	1.57
	450.000	470.687 (±6.904)	4.60
Freeze and thaw[c]	18.000	17.884 (±0.366)	−0.64
	450.000	462.273 (±8.771)	2.73
Long-term[d]	18.000	16.233 (±0.801)	−0.37
	150.000	135.129 (±3.583)	−9.91
	450.000	391.030 (±4.866)	−13.10

[a] After 24 h in autosampler at 5 °C.
[b] After 6 h at room temperature.
[c] After 3 freeze and thaw cycles at −70 °C.
[d] At −70 °C for 98 days.

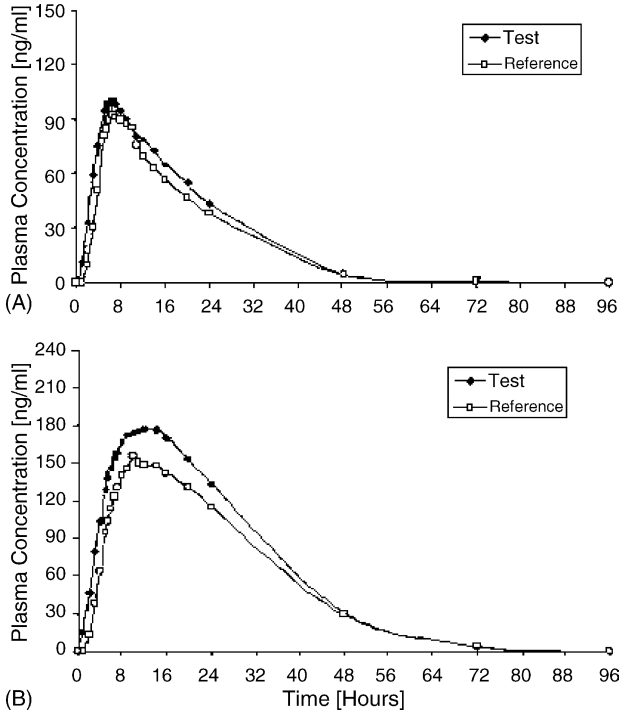

Fig. 5. Mean VEN (A) and ODV (B) plasma concentration–time profiles following a 150 mg oral dose of venlafaxine to human subjects.

male volunteers in each phase. The mean (±S.D.) plasma maximum concentration obtained for VEN in test and reference formulation is 105.72 (±41.47) ng/ml and 98.0 (±43.37) ng/ml, respectively. The mean (±S.D.) plasma maximum concentration obtained for ODV in test and reference formulation is 186.76 (±43.14) ng/ml and 162.02 (±38.72) ng/ml, respectively. The mean VEN and ODV plasma concentration–time profile following a 150 mg oral dose of venlafaxine to human subjects is shown in Fig. 5. The AUC measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from 0 to infinity, which indicates suitability of the LOQ of analytical method for pharmacokinetic studies.

4. Conclusions

A simple, specific, rapid and sensitive analytical method for the determination of VEN and ODV in human plasma has been developed. The proposed method is the first LC–MS–MS method for simultaneous determination of VEN and its active metabolite ODV. The method provided excellent specificity and linearity with a limit of quantification of 3.0 ng/ml for VEN and 6.0 ng/ml of ODV.

References

[1] E.A. Muth, J.T. Haskins, J.A. Moyer, G.E.M. Husbands, S.T. Nielsen, E.B. Sigg, Biochem. Pharmacol. 35 (1986) 4493.
[2] E.A. Muth, J.T. Haskins, J.A. Moyer, G.E.M. Husbands, T.H. Andree, Drug Dev. Res. 23 (1991) 191.
[3] M.W. Rudorfer, W.Z. Potter, Drugs 37 (1989) 713.
[4] J.A. Moyer, E.A. Muth, J.T. Haskins, E.B. Sigg, Soc. Neurosci. 10 (1984) 262.
[5] S.F. Sisenwine, J. Politowski, K. Birk, G. White, M. Dyroff, Acta Pharmacol. Toxicol. 59 (Suppl. 5) (1986) 312.
[6] J.T. Haskins, J.A. Moyer, E.A. Muth, S.T. Nielson, E.B. Sigg, Eur. J. Pharmacol. 115 (1985) 139.
[7] J.A. Moyer, E.A. Muth, T.H. Andree, Soc. Neurosci. 12 (1986) 473.
[8] S. Cherkaoui, S. Rudaz, J.L. Veuthey, E. Varesio, Electrophoresis 22 (2001) 3308.
[9] S. Cherkaoui, S. Rudaz, J.L. Veuthey, E. Varesio, Electrophoresis 22 (2001) 491.
[10] C. Desiderio, Z. Aturki, S. Fanali, Electrophoresis 22 (2001) 535.
[11] S. Fanali, S. Rudaz, J.L. Veuthey, C. Desiderio, J. Chromatogr. A 919 (2001) 195.
[12] S. Rudaz, C. Stella, S. Fanali, J.L. Veuthey, E. Balant-Gorgia, J. Pharm. Biomed. Anal. 23 (2000) 107.
[13] S. Rudaz, J.L. Veuthey, C. Desiderio, S. Fanali, Chromatografia 50 (1999) 369.
[14] S. Fanali, R. Porra, V. Cotichini, J. Capillary Electrophor. 4 (1997) 21.
[15] L. Labat, M. Deveaux, P. Dallet, J.P. Dubost, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 773 (2002) 17.
[16] R.L. Vu, D. Helmeste, L. Albers, C. Reist, J. Chromatogr. B 703 (1997) 195.
[17] H.D. Li, D.R. Ding, B.K. Zhang, H.Y. Yuan, Studies on determination of the new drug venlafaxine in human plasma by HPLC with fluorimetric detection, 2001.
[18] E.M. Clement, J. Odontiadis, M. Franklin, J. Chromatogr. B Biomed. Sci. Appl. 705 (1998) 303.
[19] M. Matoga, F. Pehourcq, K. Titier, F. Dumora, C. Jarry, J. Chromatogr. B Biomed. Sci. Appl. 760 (2001) 213.
[20] G. Tournel, N. Houdret, V. Hedouin, M. Deveau, D. Gosset, M. Lhermitte, J. Chromatogr. B Biomed. Sci. Appl. 761 (2001) 147.
[21] D.R. Hicks, D. Wolaniuk, A. Russell, N. Cavanaugh, M. Kraml, Ther. Drug Monit. 16 (1994) 100.