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Liquid chromatography tandem mass spectrometry assay for the simultaneous determination of venlafaxine and *O*-desmethylvenlafaxine in human plasma and its application to a bioequivalence study

Bhavin N. Patel[a,b], Naveen Sharma[b], Mallika Sanyal[c], Pranav S. Shrivastav[a,*]

[a] *Chemistry Department, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380009, Gujarat, India*
[b] *Analytical Laboratory, BA Research India Ltd., Bodakdev, Ahmedabad 380054, India*
[c] *Chemistry Department, St. Xaviers' College, Navrangpura, Ahmedabad 380009, India*

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ABSTRACT

A rapid, simple and sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for simultaneous determination of venlafaxine (VEN) and its active metabolite, *O*-desmethylvenlafaxine (ODV) in human plasma was developed using nadolol as internal standard (IS). The analytes and IS were extracted from 200 μl aliquots of human plasma via protein precipitation using 0.43% formic acid in acetonitrile and separated on a Hypurity cyano (50 mm × 4.6 mm, 5 μm) column. Quantitation was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique, operating in multiple reaction monitoring (MRM) and positive ion mode. The precursor to product ion transitions monitored for VEN, ODV and IS were *m*/*z* 278.3 → 58.1, 264.3 → 58.1 and 310.4 → 254.1, respectively. The total chromatographic runtime was 3 min with retention time for VEN, ODV and IS at 1.93, 1.50 and 1.29 min, respectively. The method was fully validated for its sensitivity, accuracy and precision, linearity, recovery, matrix effect, dilution integrity and stability studies. The linear dynamic range of 2.0–500 ng/ml was established for both VEN and ODV with mean correlation coefficient (*r*), 0.9994 and 0.9990, respectively. The intra-batch and inter-batch precision (%CV) in three validation batches across five concentration levels (LLOQ, LQC, MQC, HQC and ULOQ) was less than 12.6% for both the analytes. The accuracy determined at these levels was within −9.8 to +3.9% in terms of %bias. The method was successfully applied to a bioequivalence study of 150 mg venlafaxine extended release capsule formulation in 22 healthy Indian male subjects under fed condition.

1. Introduction

Venlafaxine {1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol, VEN} is a second generation antidepressant drug, which selectively inhibits re-uptake of norepinephrine and serotonin, with weak inhibition of dopamine re-uptake [1,2]. It is structurally novel phenethylamine bicyclic derivative, chemically unrelated to tricyclic, tetracyclic or other available antidepressant agents [3,4]. The mechanism of venlafaxine's antidepressant actions in humans is believed to be associated with its potentiation of neurotransmitter activity in the central nervous system. VEN does not have significant affinity for muscarinic, histaminergic or α_1-adrenergic receptors in vitro [5]. In humans, it is metabolized in liver by cytochrome P450 (CYP) enzymes to one major active metabolite *O*-desmethylvenlafaxine (ODV, 56%) and two other minor metabolites *N*,*O*-didesmethylvenlafaxine (DDV, 16%) and *N*-desmethylvenlafaxine (NDV, 1%), respectively [6]. ODV significantly contributes towards the therapeutic effect of VEN, since it is found in plasma at high concentration [7] and has a long half life (11–13 h) [8].

VEN is rapidly and extensively distributed throughout the body. It binds only weakly to plasma proteins (~27%), similarly the plasma protein binding of ODV is ~30%. Hence no drug interaction involving plasma protein is anticipated. The pharmacokinetics of both VEN and ODV are linear over the dose range of 75–450 mg per day (administered every 8 h). There is no significant change in their T_{max}, half-life, volume of distribution or clearance with dose, while C_{max} and AUC increase proportionately with dose [9].

Many methods for screening of drugs including venlafaxine for toxicology studies and therapeutic drug monitoring have been described [10–15]. Methods capable of separating enantiomers of venlafaxine and *O*-desmethylvenlafaxine have been reported based on capillary electrophoresis [16–19] and HPLC-ESI/MS [20]. Recently, Wei et al. [21] have determined only venlafaxine in

* Corresponding author. Tel.: +91 79 26300969; fax: +91 79 26308545.
E-mail address: pranav_shrivastav@yahoo.com (P.S. Shrivastav).

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human plasma by LC–MS with a sensitivity of 1.0 ng/ml employing liquid–liquid extraction (LLE) with ether. Since VEN and ODV have similar pharmacological activity, it is important to monitor the plasma concentration of both for pharmacokinetic studies. Several methods have been presented for the simultaneous determination of VEN and ODV in human plasma. Theron et al. [22] have demonstrated the importance of atmospheric pressure photoionization (APPI) in LC–MS to minimize ion suppression and matrix effect for the quantification of venlafaxine and *O*-desmethylvenlafaxine in human plasma. Mainly conventional HPLC with UV [6,23], fluorescence [24,25] and coulometric detection [26] have been used in their simultaneous determination. The UV method reported by Matoga et al. [23] has a very poor sensitivity (200 ng/ml) and a high chromatographic run time. Both the methods on HPLC-fluorescence are adequately sensitive but the turnaround time for analysis is very high. Also, one of these methods [24] uses very high injection volume (100 μl) in HPLC system and relatively large plasma volume (1 ml) for sample preparation. Apart from these five methods, two other methods [27,28] describe the application of mass spectrometry and merit comparison with the present work. Bhatt et al. [27] have reported a rapid method (3 min) using solid phase extraction (SPE) employing 0.5 ml plasma volume for processing with a sensitivity of 3.0 and 6.0 ng/ml for VEN and ODV, respectively. A promising method was developed and validated by Liu et al. [28] for venlafaxine and its three metabolites in human plasma using liquid–liquid extraction with ether followed by MS detection in linear dynamic range of 4.0–700 and 2.0–900 ng/ml, respectively. Although the method is sensitive and has an efficient extraction procedure but the total chromatographic run time is too long (10 min), which may not be favourable for routine subject analysis. Also, all these reported procedures have a very high on-column loading of the analytes at the ULOQ level, which may reduce the efficiency of the column and may affect the column life.

Thus, the aim of the present work was to establish a simple, accurate, rapid and sensitive method using LC–MS/MS for the simultaneous quantification of venlafaxine and *O*-desmethylvenlafaxine in human plasma. The paper describes a simple and efficient sample extraction via protein precipitation employing small plasma volume (200 μl) for processing and a short chromatographic run time (3 min) for the separation of both the analytes and IS. This sensitive (2 ng/ml) method was successfully applied to a pharmacokinetic study of 150 mg venlafaxine extended release capsule formulation in 22 healthy Indian male subjects under fed condition.

2. Experimental

2.1. Chemicals, materials and reagents

Reference standard material of venlafaxine hydrochloride (98.9%) and *O*-desmethylvenlafaxine (98.4%) were procured from Cadila Healthcare Ltd. (Ahmedabad, India), while nadolol (IS, 98.8%) was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from S.D. Fine Chemicals Ltd. (Mumbai, India). Ammonium trifluoroacetate used in mobile phase was of Acros Organics (New Jersey, USA) while AR grade formic acid was obtained from S.D. Fine Chemicals Ltd. (Mumbai, India). Water used for the LC–MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, India). Control buffered (K3 EDTA) human plasma was obtained from Clinical Department, BA Research India Limited (Ahmedabad, India) and was stored at −20 °C. Centrifuge was of Eppendorf 5810 (Hamburg, Germany) having maximum speed of 20820 × *g*.

Dilution solution consisted of 500 ml methanol + 500 ml deionized water + 0.80 ml formic acid.

2.2. LC–MS/MS instrumentation and conditions

The liquid chromatography system (Shimadzu, Kyoto, Japan) consisted of a LC-10ADvp pump, an autosampler (SIL-HTc) and an on-line degasser (DGU-14A). Chromatographic column used was Hypurity cyano, 50 mm length × 4.6 mm inner diameter, with 5.0 μm particle size. The mobile phase consisted of 350 ml methanol + 650 ml deionized water + 1.5 ml, 1.0 M ammonium trifluoroacetate. Separation of analytes and IS was performed under isocratic condition at a flow rate of 700 μl/min. The auto sampler temperature was maintained at 4 °C and the injection volume was 5 μl. The total LC run time was 3 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-3000 equipped with Turbo Ion spray®, manufactured by MDS SCIEX (Toronto, Canada) operating in the positive ion mode. Quantitation was done using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transition of *m*/*z* 278.3 → 58.1 for venlafaxine, 264.3 → 58.1 for *O*-desmethylvenlafaxine and 310.4 → 254.1 for IS (Fig. 1). All the parameters of LC and MS were controlled by Analyst software version 1.4.1.

For venlafaxine, *O*-desmethylvenlafaxine and nadolol (IS) the source parameters maintained were nebuliser gas (NEB):12 psi, ion spray voltage (ISV): 2000 V, turbo heater temperature (TEM): 550 °C, entrance potential (EP): 10 V, collision activation dissociation (CAD): 6 psi, curtain gas (CUR): 12 psi. The compound dependent parameters like declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were optimized at 26, 220, 35 and 10 V for venlafaxine, 26, 200, 45 and 10 V for *O*-desmethylvenlafaxine and 26, 200, 25 and 16 V for nadolol, respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 600 ms for both the analytes and 200 ms for IS.

2.3. Preparation of standard stocks and plasma samples

The standard stock solutions of 100 μg/ml were prepared by dissolving requisite amount of venlafaxine, *O*-desmethylvenlafaxine and nadolol (IS) in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 10 μg/ml for both the analytes. Combined working solutions of venlafaxine and *O*-desmethylvenlafaxine, required for spiking plasma calibration and quality control samples were subsequently prepared using the standard and intermediate stock solutions in methanol:water (50:50, v/v). IS working solution of 50 ng/ml was prepared using the stock of 100 μg/ml in 0.43% formic acid in acetonitrile. All the standard stock, intermediate stock and working stock solutions were prepared and stored at 4 °C (±6 °C) until used. Drug free plasma, i.e. control (blank) plasma was withdrawn from the deep freezer and allowed to completely thaw before use. The calibration standards (CS) and quality control (QC) samples (LLOQ, lower limit of quantitation; LQC, low quality control; MQC, middle quality control; HQC, high quality control; ULOQ, upper limit of quantitation) were prepared by spiking blank plasma with respective working solutions (5% of total volume of plasma). Calibration standards were made at 2.0, 4.0, 10.0, 20.0, 40.0, 100, 200, 400, and 500 ng/ml for both the analytes. Quality controls were prepared at 2.0 ng/ml (LLOQ), 5.0 ng/ml (LQC), 45.0 ng/ml (MQC), 375 ng/ml (HQC) and 500 ng/ml (ULOQ) for VEN and ODV. The spiked plasma samples at all the levels were stored at −20 °C for validation and subject sample analysis.

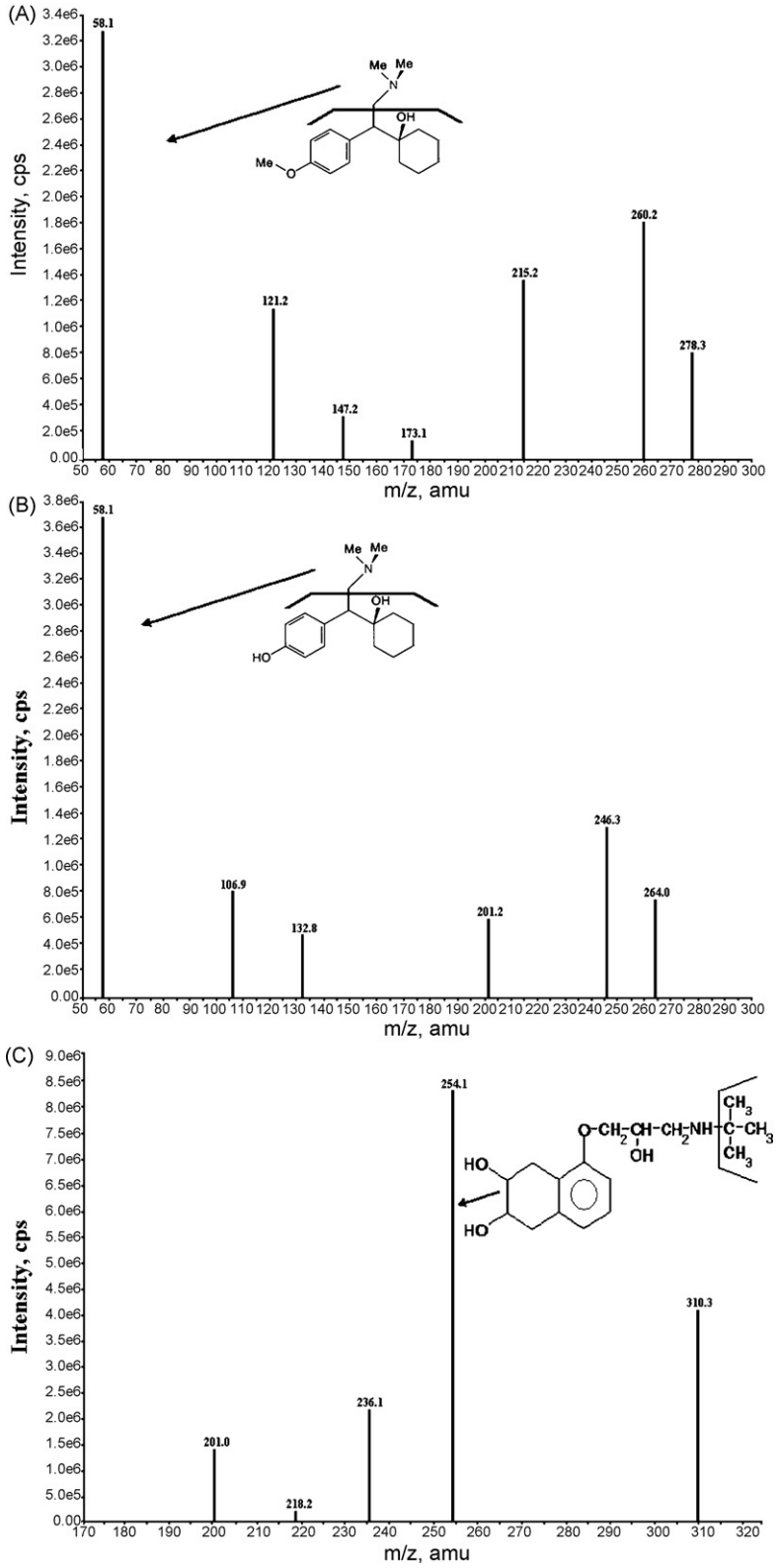

Fig. 1. Product ion spectra of (A) venlafaxine (B) *O*-desmethylvenlafaxine and (C) nadolol (IS).

2.4. Procedure for sample extraction

Prior to analysis, spiked plasma samples were withdrawn from −20 °C freezer and thawed for 30–45 min at room temperature. The samples were vortexed adequately using a vortex mixer before pipetting. Aliquots of 200 μl plasma were transferred into polypropylene micro-centrifuge tubes, 10 μl deionized water along with 500 μl working solution of IS (50.0 ng/ml) was added. The tubes were capped and vortexed vigorously for 60 s, followed by centrifugation for 10 min at 17,950 × *g*. 200 μl of supernatant was

added to 1000 μl of dilution solution and mixed thoroughly and 5 μl was used for injection in LC–MS/MS, in partial loop mode.

2.5. Methodology for validation

A thorough and complete method validation of venlafaxine and *O*-desmethylvenlafaxine in human plasma was done following the USFDA guidelines [29]. The method was validated for selectivity, sensitivity, interference check, linearity, precision and accuracy, recovery, matrix effect, cross-specificity, stability and dilution integrity.

Test for selectivity was carried out in 10 different lots of blank plasma (with K3 EDTA as anticoagulant), processed by the same protein precipitation extraction protocol and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and the internal standard (IS). In this experiment, from each of these 10 different lots, two replicates each of 190 μl were spiked with 10 μl methanol–water solution (50:50, v/v). In the first set, the blank plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with only IS before extraction (total 20 samples). Further, one system suitability sample (SSS) at CS-2 concentration and two replicates of LLOQ concentration (CS-1) were prepared by spiking blank plasma with combined working aqueous standards of VEN and ODV (5% of total volume of plasma). The blank plasma sample used for spiking of SSS and LLOQ were chosen from one of these 10 lots of plasma. Check for interference due to commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 μg/ml) were prepared by dissolving requisite amount in methanol. Further, working solutions (100 ng/ml) were prepared in the mobile phase and 5 μl was injected to check any possible interference at the retention time of analytes and IS.

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Five linearity curves containing nine non-zero concentrations were analysed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$) for VEN and ODV. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the drugs in plasma over the range tested.

Intra-batch and inter-batch accuracy and precision was evaluated at five different concentrations levels (LLOQ, LQC, MQC, HQC and ULOQ) in six replicates for both the analytes. Mean values were obtained for calculated drug concentration over these batches. The accuracy and precision was calculated and expressed in terms of %bias and coefficient of variation (%CV), respectively.

Recovery of the analytes from the extraction procedure was performed at LQC, MQC and HQC levels. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

To study the effect of matrix on analyte quantitation with respect to consistency in signal (suppression/enhancement), matrix effect was checked in six different lots of K3 EDTA plasma. Four replicates, each at LQC and HQC levels were prepared from these lots of plasma (total 48 QC samples) and checked for the accuracy in terms of % bias in all the QC samples. Cross specificity experiment were conducted for VEN, ODV and IS at ULOQ level by comparing the peak area at their respective retention times.

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability sample of analytes and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability (BTS), room temperature stability (SBM), refrigerated stability of extracted sample (RSS), freeze thaw stability (FTS) and long term stability (LTS) were performed at LQC, MQC and HQC levels using six replicates at each level.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 5 times the ULOQ concentration, i.e. 2500 ng/ml for venlafaxine and *O*-desmethylvenlafaxine and also at HQC level for both the analytes. Six replicate samples each of 1/10 of 5× ULOQ (250.0 ng/ml) and 1/10 of HQC (37.5 ng/ml) concentration were prepared by adding blank plasma and their concentrations were calculated, by applying the dilution factor of 10 against the freshly prepared calibration curve for VEN and ODV.

2.6. Bioequivalence study

The design of study comprised of "An open label, randomized, two period, two treatment, two sequence, single dose, crossover study, balanced, comparative evaluation of relative bioavailability of test (150 mg venlafaxine hydrochloride extended release capsules) and reference formulation (EFFEXOR XR, 150 mg venlafaxine hydrochloride capsules) in 22 healthy Indian subjects under non-fasting conditions". All the subjects were informed of the aim and risk involved in the study and written consent were obtained. Ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [30]. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBSAg and HCV tests. They were orally administered a single dose of test and reference formulation after recommended wash out period with 240 ml of water. Drinking water was not allowed and supine position was restricted 2 h post-dose. Standardized meals were provided as per schedule, which included milk, toast, onion, peanuts and vegetable cutlets in oil. The meal contained 62.6 g fat, 33.1 g proteins and 62.4 g carbohydrates, giving total energy of 0.95 kcal. Blood samples were collected in vacutainers containing K3 EDTA before (0 h) and at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 14.0, 16.0, 24.0, 36.0, 48.0, 60.0 and 72.0 h of administration of drug. Blood samples were centrifuged at 2060 × *g* for 10 min and plasma was separated, stored at −20 °C until use.

3. Results and discussion

3.1. Method development

This bioanalytical method was developed and validated for assaying venlafaxine and *O*-desmethylvenlafaxine in therapeutic concentration range for the analysis of routine samples. The therapeutic plasma levels of VEN usually are in the range 30–200 ng/ml, while for ODV it is between 50 and 500 ng/ml [31]. Thus it was important to develop a simple, rapid and accurate method for simultaneous extraction of VEN and ODV from human plasma. Prime consideration was given to sample processing, chromatographic separation and setting of mass parameters for optimum response.

During method development, tuning of MS parameters in positive and negative ionization modes was carried out for VEN, ODV and nadolol (IS) using 1000 ng/ml tuning solution. However, the response observed was much higher in positive ionization mode for all three compounds compared to the negative mode due to their basic nature. Moreover, use of ammonium trifluoroacetate (1.0 M) in the mobile phase further enhanced the response for both the analytes and IS with low background noise, resulting in higher sensitivity. The analytes and IS gave predominant singly charged protonated precursor $[M+H]^+$ ions at m/z of 278.3, 264.3 and 310.4 for VEN, ODV and IS, respectively, in Q1 MS full scan spectra. Further, fragmentation was initiated using sufficient nitrogen for collision activated dissociation and by applying 15 V collision energy to break the precursor ions. However, the most abundant ions found in the product ion mass spectra were m/z 58.1, 58.1 and 254.1 at 35, 45 and 25 V collision energy for VEN, ODV and IS, respectively. To attain an ideal Taylor cone and a better impact on spectral response, nebuliser gas pressure was optimized at 12 psi due to the high flow rate (700 μl/min). Fine tuning of nebuliser gas and CAD gas was done to get a consistent and stable response. It was observed that ion spray voltage had a significant effect on the response of both the analytes and IS. At high voltage (4500 V), the response was drastically reduced and hence an optimum potential of 2000 V was kept which gave consistent and stable signal. The ion source chamber temperature had little effect on the signal and hence was maintained at 550 °C. A dwell time of 600 ms was adequate and no cross-talk was observed between the MRMs of analytes.

Most of the reported procedures [23–28] have applied solid phase extraction or liquid–liquid extraction for plasma extraction with quantitative recoveries. Thus, initially both these extraction methodologies were tried using Oasis HLB cartridges for SPE and different organic solvents like hexane, dichloromethane, diethyl ether and methyl tert-butyl ether (MTBE) for LLE. The results obtained were comparable with the reported procedures in terms of quantity and consistency in recovery, but as the purpose was to develop a simple, quick and inexpensive method, protein precipitation (PP) was tested. Also, there are no reports of protein precipitation for simultaneous extraction of VEN and ODV in the literature so far. Thus, in the present study PP was carried out using ethanol, methanol, acetone and acetonitrile solvents. The extracts were clear but the recovery was in the range of 60–70% for all the solvents. Addition of formic acid to these solvents in different volume ratios helped in obtaining consistent and reproducible response. The best results were obtained with 0.43% (v/v) formic acid in acetonitrile. The method gave clear extracts with minimum matrix effect and quantitative extraction was possible for both the analytes and IS. The mean recoveries for VEN, ODV and IS obtained were 94.7, 93.2 and 101.0%, respectively. Moreover, the validation results and subject sample analysis study support this extraction methodology and hence was accepted in the present study.

Chromatographic conditions were optimized to obtain sharp peak shape with adequate response. This included mobile phase selection, pH of buffer solution, flow rate, column type and injection volume. Different volume ratios of methanol–water and acetonitrile–water combinations were tried as mobile phase, along with ammonium trifluoroacetate, ammonium acetate and ammonium formate buffers in varying strength on Aquasil C18 (100 mm × 2.1 mm i.d., 5 μm), Hypurity cyano (50 mm × 4.6 mm i.d., 5 μm) and Betabasic cyano (100 mm × 2.1 mm i.d., 5 μm). It was observed that 1.5 ml, 1 M ammonium trifluoroacetate in methanol:water (35:65, v/v) as the mobile phase was most appropriate for faster elution, better efficiency and peak shape. The use of Hypurity cyano chromatography column helped in the separation and elution of all three compounds in a very short time. The maximum on-column (at ULOQ level) loading of VEN and ODV per sample injection volume (5 μl) was only 119 pg. The total chromatographic run time was 3 min for each run at 700 μl/min flow rate.

As per USFDA guideline, an ideal internal standard should mimic the analyte in as many ways as possible. It should have a similar structure, same physicochemical properties or can be a labeled compound. Nadolol though belonging to a different class of compounds but with some structural similarity with the analytes was tested as an internal standard. The results were encouraging as all three compounds had similar chromatographic behavior and were easily extracted from plasma proteins with 0.43% formic acid in acetonitrile. Moreover, there was no significant matrix effect of IS on both the analytes. Also, the validation results obtained from this LC–MS/MS methodology prompted its selection as an IS for the present study.

3.2. Selectivity, sensitivity (LLOQ) and interference check

The aim of performing selectivity check with 10 different types of plasma samples was to ensure the authenticity of the results for study sample analysis. Figs. 2 and 3 demonstrate the selectivity results with the chromatograms of double blank plasma (without IS), blank plasma (with IS), and the peak response of VEN and ODV at LLOQ (2.00 ng/ml for both) concentration. The protein precipitation methodology in combination with mass spectrometry detection gave very good selectivity for the analytes and IS. The chromatograms show excellent peak shape for both the analytes and IS. No endogenous interferences were found at the retention times of VEN (1.93 min), ODV (1.50 min) and IS (1.29 min) in the blank plasma. The retention time was short for both the analytes, which makes it suitable for routine analysis. The area observed at the retention time of VEN and ODV was less than 20% of their LLOQ area whereas, it was less than 5% IS area observed in the LLOQ sample. Negligible (<0.1%) interference was observed for commonly used medications by subjects like paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen, which is evident from the ion chromatograms at LLOQ level and real subject sample chromatograms of VEN and ODV at 8.0 h and 11.0 h, respectively.

3.3. Linearity, accuracy and precision

The nine point calibration curves for VEN and ODV were linear from 2.0 to 500 ng/ml. The equations for means ($n = 5$) of five calibration curves for each analyte were: venlafaxine; $y = 0.01378x + 0.00113$ ($r = 0.9994$), *O*-desmethylvenlafaxine; $y = 0.01222x + 0.00256$ ($r = 0.9990$). The standard deviation values obtained (slope, intercept) from five regressions were (0.001, 0.001) and (0.000, 0.002) for VEN and ODV, respectively.

The intra-assay precision and accuracy were evaluated in six replicate analyses for VEN and ODV at five concentration levels viz. LLOQ, LQC, MQC, HQC and ULOQ each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. Concentrations were calculated from the calibration curve and the intra-batch and inter-batch precision (%CV) ranged from 2.4 to 7.0% and 1.1 to 12.6% for VEN and ODV, respectively. The accuracy expressed in terms of % bias was within −9.8 to +3.9% for both the analytes.

3.4. Recovery and matrix effect

The overall mean recovery for VEN at LQC, MQC and HQC levels were 89.3, 95.4 and 99.5% and that for ODV was 90.1, 91.9 and 97.5%, respectively with variability (%CV) between them of 5.4 and 4.1% for VEN and ODV, respectively. The recovery of IS was found

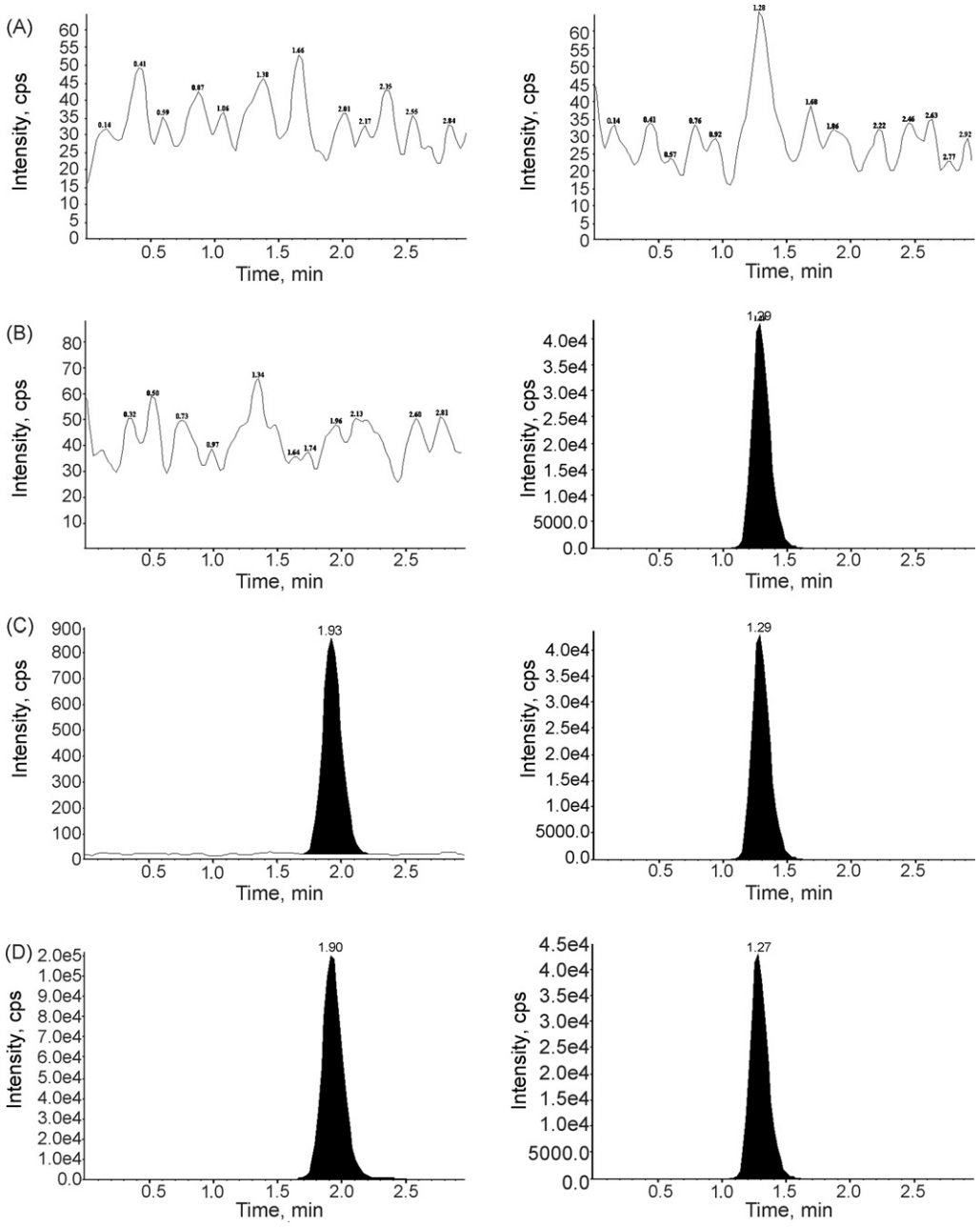

Fig. 2. Chromatograms for venlafaxine (278.3 → 58.1) in double blank plasma (A), blank + IS (B), LLOQ (C) and real subject sample at 8.0 h (D).

to be 101.0%. Thus, the consistency in recoveries of VEN, ODV and IS supports the extraction procedure for its application to routine sample analysis.

Matrix effect is due to co elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response 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 for supporting pharmacokinetics studies. Assessment of matrix effect was done with the aim to see the effect of different lots of plasma on the back-calculated value of QC's nominal concentration. The results found were well within the acceptable range as shown in Table 1. Ion suppression was checked by comparing the area response of unextracted samples to that of aqueous sample. Unextracted samples were prepared in six different lots of blank plasma. Ion suppression was minor in all the lots and it was consistent as CV of area ratio (unextracted/aqueous) was less than 5%. Moreover, the minor suppression of analyte signal due to endogenous matrix does not affect the quantitation of analytes and IS peak. Cross specificity experiment indicated no interconversion between VEN and ODV, as the area observed at the retention time of VEN in presence of ULOQ area of ODV and vice-versa was negligible. Also, the extraction method was rugged enough and gave accurate and consistent results when applied to real patient samples.

3.5. Stability and dilution integrity

The stability experiments were performed thoroughly to evaluate their stability in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was com-

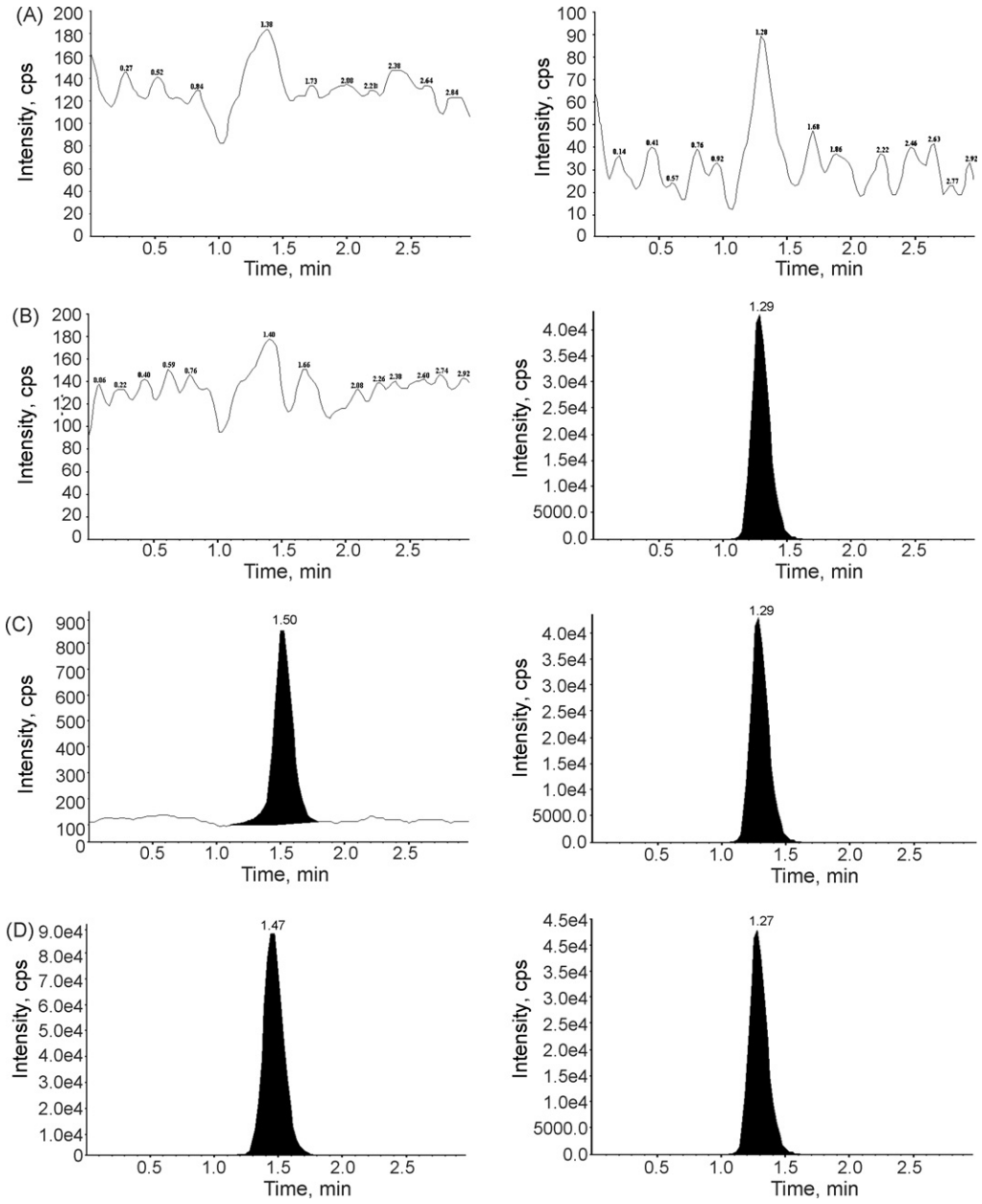

Fig. 3. Chromatograms for *O*-desmethylvenlafaxine (264.3 → 58.1) in double blank plasma (A), blank + IS (B), LLOQ (C) and real subject sample at 11.0 h (D).

Table 1
Matrix effect in human plasma at LQC and HQC levels

Plasma lot	LQC (5.0 ng/ml)		HQC (375 ng/ml)	
	Mean calculated conc.[a]	%Bias	Mean calculated conc.[a]	%Bias
Venlafaxine				
Lot-1	4.8	−4.0	347	−7.5
Lot-2	5.1	2.0	368	−1.9
Lot-3	4.9	−2.0	358	−4.5
Lot-4	4.9	−2.0	384	2.4
Lot-5	4.9	−2.0	357	−4.8
Lot-6	4.9	−2.0	364	−2.9
O-Desmethylvenlafaxine				
Lot-1	4.8	−4.0	351	−6.4
Lot-2	4.9	−2.0	370	−1.3
Lot-3	4.9	−2.0	353	−5.9
Lot-4	5.1	2.0	378	0.8
Lot-5	4.8	−4.0	357	−4.8
Lot-6	4.8	−4.0	363	−3.2

[a] Mean of four replicates for each lot.

pared with freshly prepared quality control samples. The results obtained were well within the acceptable limits. Stock solutions of VEN and ODV were stable at room temperature for 7 h and at 4 °C (±6 °C) for 25 days with mean % change well within ±3.5%. The intermediate solution of VEN and ODV in methanol–water (50:50, v/v) was stable for 24 days. Both the analytes were found stable in controlled plasma at room temperature upto 24 h and for at least six freeze and thaw cycles. The analytes in extracted plasma samples were stable for 96 h under refrigerated condition of 4 °C (±6 °C). Bench-top stability of extracted samples was also up to 96 h. The VEN and ODV spiked plasma samples stored at −20 °C for long term stability were found stable for minimum period of 64 days. The values for the percent change for the above stability experiments are compiled in Table 2.

The mean back-calculated concentrations for 1/10 dilution samples were within 85–115% of their nominal values. The precision (%CV) for 1/10 dilution samples was <5.0 for both the analytes.

Table 2
Stability results for venlafaxine and *O*-desmethylvenlafaxine

Stability	Storage condition	Level[a]	Venlafaxine			*O*-Desmethylvenlafaxine		
			A (ng/ml)	%CV	%Bias	*A* (ng/ml)	%CV	%Bias
Stability in biological matrix (SBM)	Room temperature (24 h)	LQC	4.7	4.3	−6.6	4.8	5.2	−4.5
		MQC	42.4	0.9	−5.7	43.0	1.1	−4.4
		HQC	370	7.7	−1.3	372	7.4	−0.9
Refrigerator stability of extracted samples (RSS)	Autosampler (4 °C, 96 h)	LQC	4.6	2.9	−8.9	4.5	3.2	−9.3
		MQC	43.0	2.8	−4.6	43.3	2.6	−3.8
		HQC	344	1.2	−8.2	346	0.8	−7.7
Bench top stability of extracted samples (BTS)	Room temperature (96 h)	LQC	4.7	3.5	−5.8	4.6	4.4	−7.6
		MQC	43.2	1.4	−3.9	42.9	2.4	−4.7
		HQC	346	2.1	−7.7	348	2.5	−7.3
Freeze and thaw stability (FTS)	After 6th cycle at −20 °C	LQC	5.2	7.6	4.0	5.1	4.1	2.2
		MQC	48.8	2.4	8.4	50.0	1.7	11.0
		HQC	402	2.4	7.3	410	2.5	9.4
Long term stability (LTS)	64 days at −20 °C	LQC	5.1	3.8	2.3	5.0	6.5	1.0
		MQC	46.6	4.4	3.6	45.1	3.9	0.1
		HQC	379	3.7	1.1	366	4.2	−2.3

A, mean comparison concentration; CV, coefficient of variance.
[a] Mean of six replicates at each level.

3.6. Bioequivalence study in human subjects

Venlafaxine hydrochloride is normally administered by the oral route. It is rapidly and extensively absorbed from the small intestine. At least 92% of oral dose is absorbed, achieving peak plasma concentration in 2–2.5 h (T_{max}) after single oral dose of 25–150 mg. The T_{max} for ODV is in the range of 4–5 h. The presence of food does not significantly affect the peak plasma concentration (C_{max}) or the area under the curve (AUC) of either venlafaxine or its metabolite [9]. The proposed validated method was applied for the assay of venlafaxine and *O*-desmethylvenlafaxine in 22 healthy Indian adult male subjects who received 150 mg test and reference formulations of venlafaxine under fed condition. The samples were processed based on the proposed extraction protocol for quantification of VEN and ODV. The method was sensitive enough to monitor their plasma concentration up to 72.0 h. In all 1164 samples including the calibration, QC and volunteer samples were run and analyzed in only 3 days and the precision & accuracy for calibration and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for the treatment, under fed condition is presented in Fig. 4. The pharmacokinetic parameters like maximum plasma concentration C_{max}, area under the plasma concentration–time curve from 0 h to the last measurable concentration AUC_{0-t}, area under the plasma concentration–time curve from 0 h to infinity $AUC_{0-\infty}$, time point of maximum plasma concentration curve T_{max}, elimination rate constant K_{el} and half life of drug elimination during the terminal phase $t_{1/2}$ were calculated for VEN and ODV. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 3. The 90% confidence interval of the individual ratio geometric mean for test/reference of VEN was within 105.9–114.6%; 105.1–113.7% and 102.9–114.3% for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, respectively. Similarly for ODV it was within 100.5–109.8%, 100.0–109.1% and 102–112.9% for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, respectively. The minimum and maximum value of T_{max} for VEN and ODV were 8.0–12.0 h (test); 5.0–10.0 h (reference) and 11.0–24.0 h (test); 8.0–24.0 h (reference), respectively. These obser-

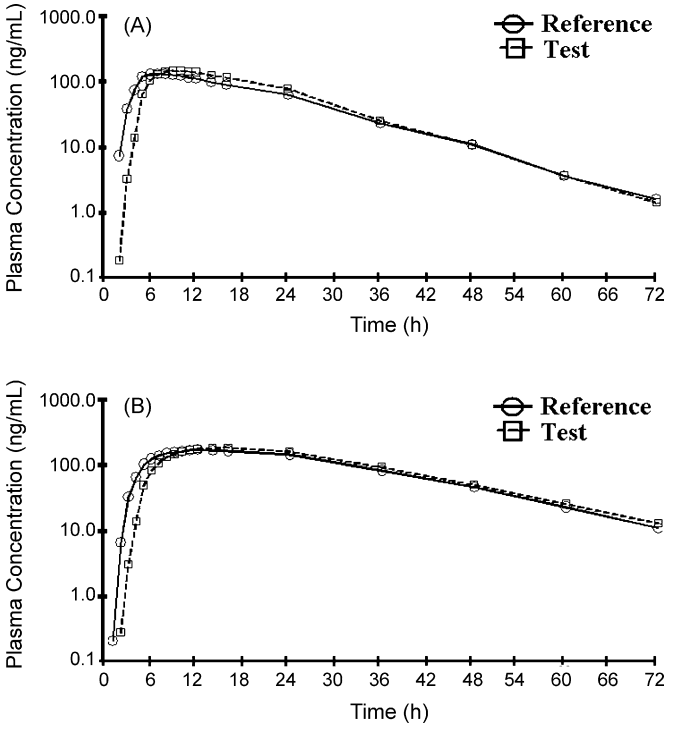

Fig. 4. Mean pharmacokinetic profile of venlafaxine and *O*-desmethylvenlafaxine after oral administration of 150 mg venlafaxine tablet to 22 healthy subjects.

Table 3
Pharmacokinetic parameters of venlafaxine and *O*-desmethylvenlafaxine in 22 healthy Indian subjects following oral dose of 10 mg venlafaxine tablet formulation

Parameter	Test (mean ± %R.S.D.)	Reference (mean ± %R.S.D.)
Venlafaxine		
C_{max} (ng/ml)	157.3 ± 32.0	144.4 ± 30.6
T_{max} (h)	9.4 ± 13.4	7.0 ± 23.3
$t_{1/2}$ (h)	8.0 ± 19.6	8.6 ± 20.3
$AUC_{0-72\,h}$ (h ng/ml)	3230.3 ± 46.2	2916.3 ± 43.2
$AUC_{0-\infty}$ (h ng/ml)	3276.7 ± 46.1	2975.9 ± 42.8
K_{el} (1/h)	0.1 ± 19.5	0.1 ± 18.3
O-Desmethylvenlafaxine		
C_{max} (ng/ml)	200.3 ± 25.2	189.4 ± 29.1
T_{max} (h)	15.6 ± 24.7	13.1 ± 25.8
$t_{1/2}$ (h)	11.8 ± 16.3	11.9 ± 16.4
$AUC_{0-72\,h}$ (h ng/ml)	6405.6 ± 27.9	6111.8 ± 28.2
$AUC_{0-\infty}$ (h ng/ml)	6647.7 ± 29.2	6388.4 ± 30.1
K_{el} (1/h)	0.1 ± 16.0	0.1 ± 17.0

vations confirm the bioequivalence of 150 mg test sample with the reference product in terms of rate and extent of absorption. These results can be compared to the study reported by Bhatt et al. [27] on Indian healthy males using sustained release 150 mg formulation of venlafaxine. The mean C_{max} value for VEN was less compared to ODV in subject samples while T_{max} and $t_{1/2}$ values were similar as reported earlier [9]. Also, AUC_{0-t} was higher than 95% of the value for $AUC_{0-\infty}$, which indicates suitability of the limit of quantitation of the method for pharmacokinetic studies. Further, there was no adverse event during the course of the study. Thus, the assay procedure for venlafaxine in plasma samples demonstrated the linearity, precision and sensitivity desired for the pharmacokinetic studies of this drug.

4. Conclusion

The objective of this work was to develop a simple, cost effective, rugged and a high throughput method for simultaneous estimation of venlafaxine and its active metabolite *O*-desmethylvenlafaxine in human plasma, especially in the absorption and elimination phase after oral administration of 150 mg formulation. The method employs a simple and inexpensive protein precipitation for sample preparation with quantitative recovery for both the analytes and IS. The sensitivity of this method is comparable with other reported methods in literature. The run time per sample analysis of 3.0 min suggests high throughput of the proposed method. The maximum on-column loading of VEN and ODV was 119 pg per injection volume of 5 μl at ULOQ level. This was considerably less compared to other reported procedures, which helps in extending the lifetime and efficiency of the column. Moreover, the limit of quantification is low enough to monitor at least five half-lives of VEN and ODV concentration with good intra- and inter-assay reproducibility (%CV) for the quality controls. From the results of all the validation parameters, the method can be useful for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples with desired precision and accuracy.

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