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Validation of an LC-MS/MS method for simultaneous quantification of venlafaxine and its five metabolites in rat plasma and its application in a pharmacokinetic study

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ABSTRACT

A sensitive, selective, and reliable LC-MS/MS method was developed and validated for simultaneous quantification of venlafaxine (VEN) and its 5 metabolites (ODV, NDV, NNDDV, OHV and NODDV) in rat plasma. The calibration ranges are 15.0 to 6000 ng/mL for VEN, 1.00 to 400 ng/mL for ODV, 5.00 to 2000 ng/mL for NDV, 1.00 to 400 ng/mL for NNDDV, 10.0 to 4000 ng/mL for OHV, and 0.200 to 20.0 ng/mL for NODDV. Briefly, 50 µL of rat plasma was extracted using liquid-liquid extraction (LLE) with methyl *tert*-butyl ether (MTBE). The analytes were separated on an Agilent SB-Phenyl (50 mm × 4.6 mm, 3.5 µm) column using a binary gradient of 0.1% formic acid in water *versus* 0.1% formic acid in acetonitrile at a flow rate of 0.8 mL/min. The method was validated following FDA guidance for bioanalytical method validation. Validated method was successfully applied to a pharmacokinetic study of VEN orally administered to rats.

1. Introduction

Venlafaxine (*DL*)-1-[2-dimethylamino-1-(4-methoxy-phenyl)-ethyl]-cyclohexanol hydrochloride, a dual serotonin-norepinephrine reuptake inhibitor, was initially launched by originator Wyeth in 1994 for the treatment of depression. In 2007, venlafaxine was the sixth most commonly prescribed antidepressant on the U.S. retail market, with about 17.2 million prescriptions. In addition to major depressive disorder (MDD) venlafaxine has also been marketed for treatment of generalized anxiety disorder, social anxiety disorder, and panic disorder. Venlafaxine (VEN), marketed as an extended release formulation (Effexor XR™), is available in dosage strengths of 37.5 mg, 75 mg, and 150 mg [1]. It is readily metabolized in the liver into its major active metabolite, O-desmthyl venlafaxine (ODV), as well as four other metabolites: rac-*N*-desmethyl venlafaxine (NDV), *D,L-N,N*-didesmethyl venlafaxine (NNDDV), 4-hydroxy venlafaxine (OHV), and rac-*N,O*-didesmethyl venlafaxine (NODDV). ODV is equipotent to VEN in *in vitro* inhibition of serotonin uptake [2]. The metabolism pathway scheme of VEN is presented in Fig. 1. Wyeth has developed the active ODV metabolite of venlafaxine. ODV received approval for major depression in 2008 and is marketed as Pristiq™.

VEN and its metabolites can be quantified using liquid chromatography (LC) for the separation of the analytes in conjunction with UV [3], fluorometric [4], and coulometric detection [5]. UV and coulometric detection methods generally have reduced sensitivity and a lack of selectivity. The direct fluorometric method using an excitation at 276 nm, is more sensitive compared to the other two techniques; however, only measurements of VEN and ODV have been reported [4].

Tandem mass spectrometry (MS/MS) is a more specific mean of detection and is highly utilized in pharmacokinetic (PK) studies. The lower limit of quantification (LLOQ) for VEN and ODV was reported to be as low as ~0.1 ng/mL in human plasma and whole blood samples [6]. Several LC-MS/MS methods were developed to quantify VEN in human tissue and plasma [7,8] and VEN and ODV in human plasma [9–12]. However, these methods were limited to only VEN and its major metabolite ODV. In one recent study, a method was developed for quantification of VEN, ODV, and two additional metabolites, NDV and NODDV, in human plasma using a solid phase extraction (SPE) method and LC-MS/MS analysis [13]. The purpose of that work was to stereoselectively quantify VEN and its metabolites. Consequently, the method had a lengthy 35-min run time to separate the enantiomers.

NDV, NNDDV, OHV, and NODDV are minor VEN *in vivo* metabolites

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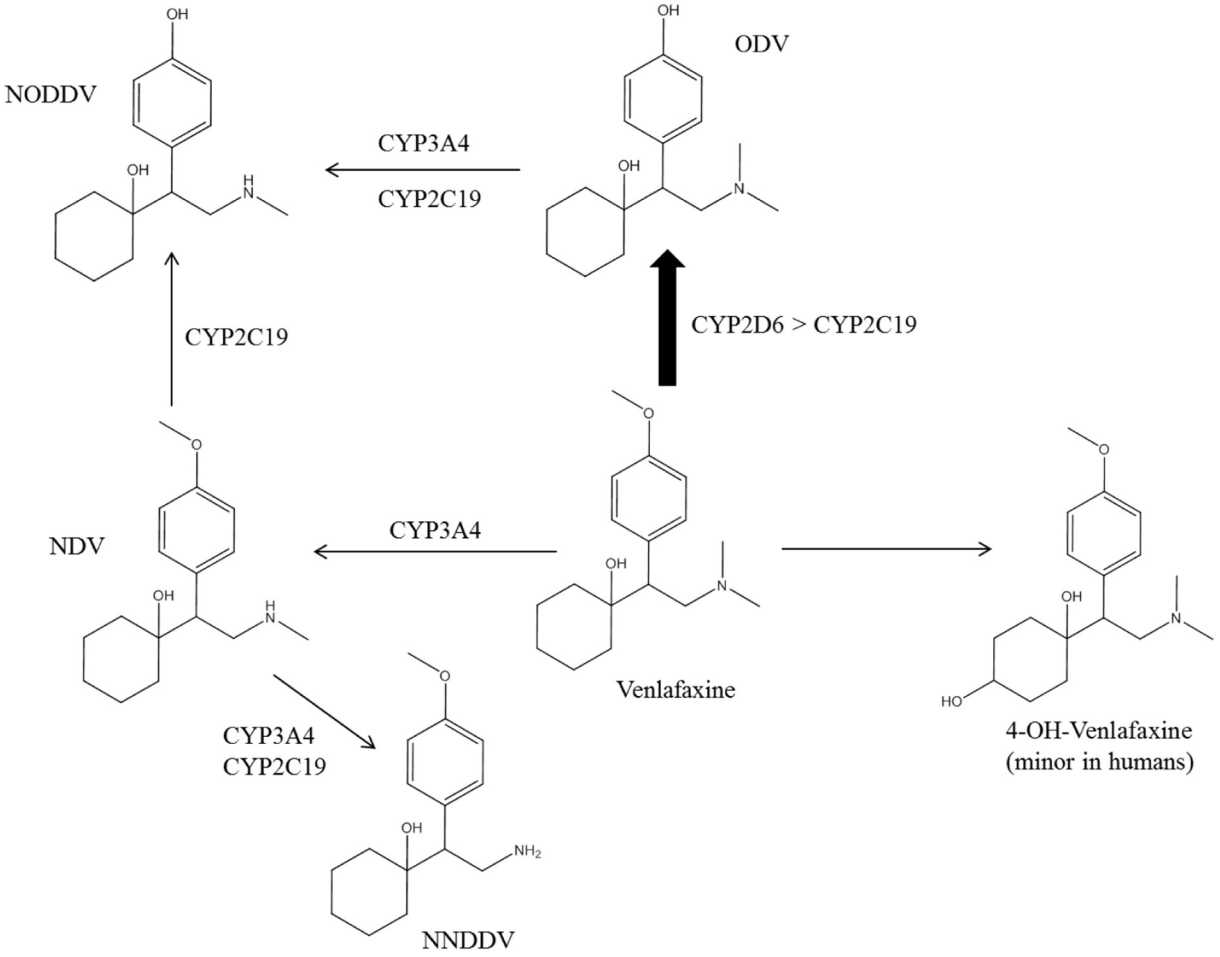

Fig. 1. Venlafaxine metabolic pathway in human.

and have not been studied extensively, but the concentration data of these metabolites will help to better understand the *in vivo* pharmacodynamic (PD) profile of VEN [14]. In order to concurrently quantify VEN, ODV, NDV, NNDDV, OHV, and NODDV in rat plasma, we developed a sensitive, selective, and robust LC-MS/MS method. The method was validated following FDA guidance for bioanalytical method validation. Satisfactory method sensitivity, selectivity, precision, and accuracy were confirmed by the validation results.

2. Materials and method

2.1. Materials and reagents

Reference standards *D,L*-venlafaxine hydrochloride (VEN), *D,L-O*-desmethyl venlafaxine (ODV), rac-*N*-desmethyl venlafaxine (NDV), *D,L-N,N*-didesmethyl venlafaxine (NNDDV), and rac-*N,O*-didesmethyl venlafaxine (NODDV) were supplied by Toronto Research Chemicals (Toronto, Ontario, Canada). Internal standards d_9-venlafaxine hydrochloride (d_9-VEN), d_6-ODV, d_6-NDV, d_3-NNDDV, and d_3-NODDV were supplied by Irvine Pharmaceutical Services (Irvine California). The reference standard for 4-hydroxy-venflafaxine (OHV) and the internal standard d_9–4-hydroxy-venlafaxine (d_9-OHV) were provided by Pharmaron (Beijing, China). Sodium hydroxide (NaOH, ACS grade), acetonitrile (ACN, HPLC grade), methyl *tert*-butyl ether (MTBE, HPLC grade), ethyl acetate (HPLC grade), and dimethyl sulfoxide (DMSO, HPLC grade) were obtained from Thermo Fisher Scientific (Fair Lawn, New Jersey). Formic acid (FA, HPLC grade) was obtained from Sigma-Aldrich Corporation (St. Louis, Missouri). K_2EDTA rat plasma and K_2EDTA rat blood were obtained from BioreclamationIVT (Westbury, New York). Ultrapure water was from a Milli-Q water purification system (Bedford, Massachusetts).

2.2. Preparation of standards and quality control samples

Stock standard solutions (1 mg/mL) of VEN, ODV, NDV, NNDDV, OHV, and NODDV were prepared by dissolving the dry compounds in DMSO. Stock standard solutions were stored at 4 °C. Working standard solutions were prepared by serial dilution of stock standard solution with ACN:water 1:1 (v:v). Working standard solutions were stored at 4 °C. Stock and working solutions for calibration standard (CS) and quality control (QC) samples were prepared separately.

The CS and QC sample were prepared as pools in rat plasma from working standard solutions at the concentrations listed in Table 1 and stored at −80 °C. The CS samples that were used for matrix stability assessments were freshly prepared on the day of the experiment.

Individual stock internal standard (IS) solutions (1 mg/mL) of d_9-VEN, d_6-ODV, d_6-NDV, d_3-NNDDV, d_9-OHV, and d_3-NODDV were prepared by dissolving the dry compounds in DMSO. The individual stock IS solutions were diluted with ACN:water 1:1 (v:v) to prepare a combined working IS solution (at a concentration of 50 ng/mL of each). The individual stock IS solutions and working IS solutions were stored at 4 °C.

2.3. Sample preparation

VEN and its metabolites were extracted using liquid-liquid extraction (LLE) with MTBE. Generally, 50.0 µL of rat plasma was spiked with 50.0 µL of combined internal standard working solution in a 96-well

Table 1
Nominal concentrations of calibration standards and quality control samples.

Sample ID		Nominal concentration (ng/mL)					
		VEN	ODV	NDV	NNDDV	OHV	NODDV
Standards (STDs)	STD-1	15	1	5	1	10	0.2
	STD-2	30	2	10	2	20	0.4
	STD-3	60	4	20	4	40	0.8
	STD-4	300	20	100	20	200	2
	STD-5	1200	80	400	80	800	4
	STD-6	2400	160	800	160	1600	8
	STD-7	4800	320	1600	320	3200	16
	STD-8	6000	400	2000	400	4000	20
Quality controls (QCs)	LLOQ-QC (Lower limit of quantification QC)	15	1	5	1	10	0.2
	LQC (low QC)	45	3	15	3	30	0.6
	MQC (medium QC)	450	30	150	30	300	6
	HQC (high QC)	4500	300	1500	300	3000	15
	DQC (dilution QC)	12,000	800	4000	800	8000	40

plate with glass insert tube. 100 μL of 25 mM NaOH was added to each sample and mixed well. 700 μL of MTBE (v:v) was then added to each sample. The samples were vortex-mixed at ~2000 rpm for ~5 min and centrifuged at ~2500 rpm for ~10 min. 200 μL of the supernatant liquid was transferred to a clean 96-well plate and evaporated under nitrogen at ~35 °C. The sample residues were reconstituted in 200 μL of ACN:water 1:9 (v:v). An aliquot of the resulted sample was then injected onto the LC-MS/MS system for analysis.

2.4. Liquid chromatography and mass spectrometry conditions

The LC-MS/MS system consisted of two Shimadzu NexeraX2 pumps and a SIL-30ACMP autosampler coupled to an AB Sciex API 5500 mass spectrometer (MS) from Sciex (Framingham, USA). Samples were ionized using electrospray (ESI) ion source in positive mode.

Chromatographic separation was achieved at ambient temperature *via* gradient elution of 0.1% FA in water (v:v) *versus* 0.1% FA in ACN (v:v) at a flow rate of 0.8 mL/min on an Agilent SB Phenyl column (50 mm × 4.6 mm, 3.5 μm). The total run time was 4.5 min. Solvent flow was diverted from the MS after the first minute of the gradient. The MS was kept in MRM scan mode with the optimal parameter settings listed in Table 2.

2.5. Data processing

The LC-MS/MS data were calculated by Analyst® software version 1.6.2 from AB Sciex (Framingham, USA). A weighted ($1/x^2$, x = concentration) linear regression was used to generate the calibration curves. The concentrations of VEN, ODV, NDV, NNDDV, OHV, and NODDV in the samples were calculated using the peak area ratio of analyte to internal standard based on the calibration curves. Mean, standard deviation, precision, accuracy, and percent difference were calculated using Microsoft Excel software.

2.6. Method validation

The method was validated according to the Guidelines of US Food and Drug Administration (FDA) bioanalytical method validation [15].

2.6.1. Precision and accuracy

The intra- and inter-day accuracy and precision (P/A) were evaluated in three batches. In each P/A batch, 3 levels of QCs (LQC, MQC, HQC) were assayed in 6 replicates along with 2 sets of calibration standards. The precision was calculated in terms of relative standard deviation (%RSD). The precision and accuracy were considered acceptable if the precision was ≤15% and the mean concentration accuracy was 85%–115% of the nominal concentration.

Table 2
Optimal mass spectrometry parameters for each compound.

Compound name	Parent ion (*m/z*)	Product ion (*m/z*)	Dwell time (msec)	Declustering potential (V)	Collision energy (V)	Entrance potential (V)	Collision cell exit potential (V)
VEN	278	173	30	60	55	10	10
ODV	264	107	30	60	70	10	10
NDV	264	147	30	60	50	10	10
NNDDV	250	215	30	60	25	10	10
OHV	294	159	30	60	33	10	10
NODDV	250	44	30	60	35	10	10
VEN-d_9 (IS for VEN)	287	176	30	60	32	10	10
d_6-ODV (IS for ODV)	270	107	30	60	38	10	10
d_6-NDV (IS for NDV)	270	150	30	60	30	10	10
d_3-NNDDV (IS for NNDDV)	253	218	30	60	25	10	10
d_9-OHV (IS for OHV)	303	216	30	60	23	10	10
d_3-NODDV (IS for NODDV)	253	47	30	60	45	10	10

2.6.2. Sensitivity and reproducibility at the LLOQ

The LLOQ-QC was assayed in 6 replicates in 3 individual batches to establish the sensitivity, precision, and accuracy at the LLOQ level. The measured concentrations were considered acceptable if they showed a precision of ≤20% and the mean concentration accuracy within 80%–120% of the nominal concentration.

2.6.3. Dilution integrity

DQCs were prepared in rat plasma and tested in 6 replicates to evaluate the dilution integrity at a 5-fold dilution. The dilution integrity was considered acceptable if the precision was ≤15% and the mean accuracy of each DQC concentration was 85%–115% of the nominal concentration.

2.6.4. Selectivity

Six lots of blank rat plasma were evaluated as blanks. The same six lots were spiked with the analytes at the LLOQ concentrations and assayed with internal standards. The results were considered acceptable if at least 5 of the 6 tested lots of blank matrix had an instrument response (peak area) of ≤20% of the response of the analytes at the LLOQ. In addition, the blank matrix must have had a peak area of ≤5% of the peak area of the internal standards. The measured concentrations of the samples spiked at the LLOQ must have had a precision of ≤20% and a mean accuracy of 80%–120% of the nominal concentration.

2.6.5. Matrix effect

Six lots of blank rat plasma were extracted and spiked post-extraction with the analytes at the LQC and HQC levels, along with the internal standards, assuming 100% recovery. For both levels (LQC and HQC), a pure standard solution (containing the analytes and internal standards) was prepared at the same concentration as the spiked blank extract for each concentration level and analyzed in triplicate. The matrix effect was assessed using internal-standard–normalized matrix factor. The internal-standard–normalized matrix factor for the 6 lots of blank matrix must have a precision of ≤15%.

2.6.6. Recovery

The LQC, MQC, HQC (6 replicates at each level), and 9 blank matrix samples were extracted according to the method. Three blank matrix extracts were spiked with the analytes and the internal standards at each concentration level equivalent to the final concentrations of the corresponding QC samples. The ratio of the mean response of extracted QC samples to the mean response of recovery samples are expressed as percent recovery. No acceptance criterion was applied to recovery; however, the extent of recovery of the analytes and the internal standards should be consistent and reproducible.

2.6.7. Carryover

To assess carryover, an extract of blank matrix was injected immediately after the highest calibration standard. The blank should demonstrate no significant response at the retention times for the analytes (≤20% of the LLOQ) and internal standards (≤5% of mean internal standard response of the calibration standards).

2.6.8. Batch size determination

One quantification batch contained extracted samples to make the batch size equivalent to any prospective study sample batch. The quantification batch included at least 2 sets of calibration standards and at least duplicate ($n = 2$) QC samples at each of the 3 levels (LQC, MQC, and HQC). The batch size was the total number of injections in the batch including system suitability samples.

2.6.9. Hemolysis effect

The effect of hemolysis on the method's precision and accuracy was evaluated. LQC samples containing 2% hemolyzed blood were assayed in 6 replicates. The mean measured concentration of the treated QC samples was considered acceptable if the accuracy was within 85%–115% of the nominal concentration, and the precision was ≤15%.

2.6.10. Stability

Stability of the stock solution (at room temperature (RT) and at 4 °C) and the working standard solutions (only at 4 °C) was determined by comparison of the stored solutions to freshly prepared solutions. The results were considered acceptable if the percent difference of the mean peak area ratios of the freshly prepared and the stored stock solution was ≤5%. The results for the stored working solutions were considered acceptable if their mean peak area ratios differed from those of freshly prepared solutions by ≤10%.

Stability of the analytes during sample collection was evaluated by spiking rat whole blood at the LQC and HQC level. The whole blood sample was split into 3 aliquots and processed to plasma immediately, after 2 h on wet ice, and after 2 h at RT. The harvested plasma samples were extracted in 3 replicates and analyzed. The results were considered acceptable if the percent difference of the mean responses between the sample immediately extracted and those stored for 2 h is within 15%.

Several experiments were conducted to evaluate the stability of the analytes when exposed to various storages or processing conditions. All stability tests included six replicates of the LQC and HQC samples and were quantified using freshly prepared calibration standards. The results were considered acceptable if the precision was ≤15% and the mean concentration accuracy was 85%–115% of the nominal concentration. Stability was assessed in extracted plasma samples after storage at 6 °C, and in plasma samples after storage at ambient temperature after freeze/thaw cycles from −80 °C to ambient temperature and after long-term storage at −80 °C by comparing them to freshly prepared calibration standards and QC samples.

2.6.11. Reinjection reproducibility

Extracted system suitability samples, matrix blank, control zero, calibration standards, and 6 replicates of LQC, MQC, and HQC samples were stored at 2 °C–8 °C for 127 h after the original analysis. The samples were then reinjected. The reinjection reproducibility was considered acceptable if the results met the batch acceptance criteria.

2.7. Pharmacokinetics of venlafaxine in rats

Six healthy female rats weighing approximately 200–300 g were used in a PK study. An oral dose of venlafaxine 200 mg/kg was given to each rat. The blood samples were drawn at 0.5, 1, 2, 4, 8, and 24 h after the administration of drug. Blood samples were collected in tubes containing dipotassium ethylenediaminetetraacetic acid (K_2EDTA) as anticoagulant and centrifuged for 10 min at approximately 2000 g at 2–8 °C. The resulting plasma samples were stored at −80 °C until analysis. The plasma samples were extracted by LLE method as described above and analyzed for VEN and five metabolites. Different PK parameters were determined using WinNonlin software.

3. Results

3.1. Sample preparation condition optimization

Two sample processing approaches, LLE and protein precipitation (PPT), were evaluated for method robustness and signal-to-noise ratio at the LLOQ level and recovery efficiency. It was found that LLE provided better signal to noise ratio at the LLOQ level compared to PPE. Meanwhile the precision and accuracy results were better at the LLOQ level for LLE method; therefore, LLE method was selected for sample preparation. The impact of the extraction solvent and pH on analyte recovery in LLE method was assessed. Two extraction solvents, ethyl acetate and MTBE, and different amounts of a pH modifier, NaOH solution, were tested. The most acceptable recovery for each analyte was

Table 3
Average calibration curve parameters for each analyte over 8 individual runs.

	Slope	Intercept	Correlation coefficient (*R*)
VEN	1.15E-03	3.42E-03	0.9970
ODV	8.23E-03	6.05E-04	0.9978
NDV	1.92E-03	1.21E-03	0.9978
NNDDV	1.51E-02	1.86E-03	0.9971
OHV	2.10E-03	4.27E-03	0.9960
NODDV	2.19E-02	5.18E-04	0.9966

observed using MTBE as the extraction solvent and 100 µL of 25 mM NaOH.

3.2. Chromatographic and mass spectrometric conditions optimization

Chromatographic and mass spectrometric conditions were optimized to obtain acceptable selectivity and sensitivity for the analytes. Because ODV and NDV share the same molecular weight (MW) and are very similar in chemical structure, it was anticipated that they would have the same optimum parent-to-product ion transition. Therefore, method development included chromatographic resolution between those 2 compounds. Similar considerations applied to NNDDV and NODDV. We tested several C18, C8, C4, and phenyl type columns and obtained satisfactory chromatographic resolution of all 6 analytes within ~2.5 min on an Agilent SB Phenyl (50 × 4.6 mm, 5 µm) column. The total run time was 4.5 min to allow for re-equilibration of the column following the gradient elution.

The ionization and fragmentation of the analytes and IS were optimized by infusion of approximately 500 ng/mL of individual stock solutions into the electron spray ionization (ESI) source of a mass spectrometer. The appropriate parent and product ions were chosen in MRM acquisition mode. Ion source parameters such as declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and entrance potential (EP) were also optimized. The optimal mass spectrometry parameters and transitions for each compound are listed in Table 2.

3.3. Method validation results

3.3.1. Linearity of the calibration curve

Calibration standards at each concentration level were analyzed in 7 individual batches. The results show that the method demonstrated acceptable linearity for the calibration standards ($R \geq 0.994$ for all analytes). Average calibration curve parameters are presented in Table 3. A representative chromatogram at ULOQ is presented in Fig. 2.

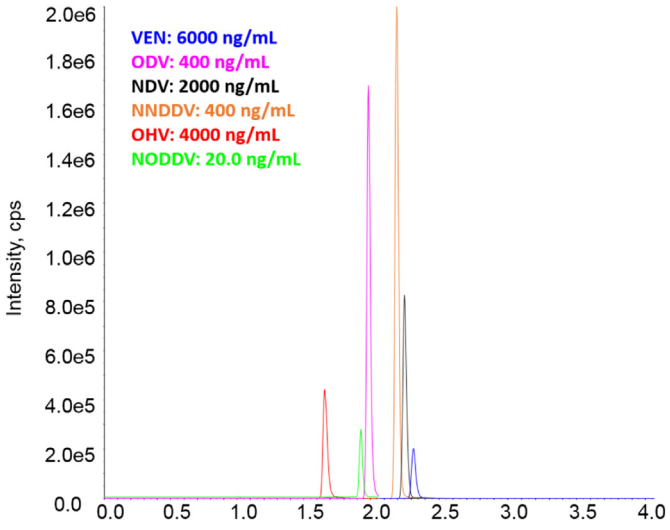

Fig. 2. Representative chromatogram at the upper limit of quantitation (ULOQ) for each analyte: Venlafaxine (VEN), *D,L*-*O*-desmethyl venlafaxine (ODV), rac-*N*-desmethyl venlafaxine (NDV), *D,L*-*N*,*N*-didesmethyl venlafaxine (NNDDV), 4-hydroxy venlafaxine (OHV), and rac-*N*,*O*-didesmethyl venlafaxine (NODDV).

Table 4
Inter-assay and intra-assay precision and accuracy ranges of quality control samples.

		Precision	Accuracy
VEN	Inter-assay	0.9%–4.7%	92.9%–102.4%
	Intra-assay	3.2%–10.1%	92.0%–105.3%
ODV	Inter-assay	2.9%–3.5%	97.3%–100.0%
	Intra-assay	2.1%–8.3%	94.0%–103.3%
NDV	Inter-assay	2.2%–5.7%	94.7%–98.0%
	Intra-assay	2.7%–14.2%	89.3%–100.7%
NNDDV	Inter-assay	3.5%–7.0%	92.3%–94.0%
	Intra-assay	2.4%–11.2%	86.0%–99.7%
OHV	Inter-assay	1.4%–2.2%	93.7%–104.7%
	Intra-assay	2.8%–7.8%	91.3%–106.0%
NODDV	Inter-assay	1.8%–7.9%	99.2%–103.3%
	Intra-assay	4.2%–8.2%	94.0%–109.3%

3.3.2. Precision and accuracy of quality control samples

The individual batch results all met the target acceptance criteria for precision and accuracy. The range for intra-assay and inter-assay precision and accuracy are presented in Table 4.

3.3.3. Sensitivity and reproducibility at the lower limit of quantification

The experimental results confirmed the sufficient sensitivity and reproducibility at the LLOQ. Precision and accuracy for all analytes ranged from 7.2%–19.6% and 83.3%–117.0% respectively.

3.3.4. Dilution integrity

Precision and accuracy of the dilution QC for all analytes ranged from 2.6%–7.0% and 97.8%–107.5% respectively. The results confirmed the acceptability of the dilution prior to sample analysis.

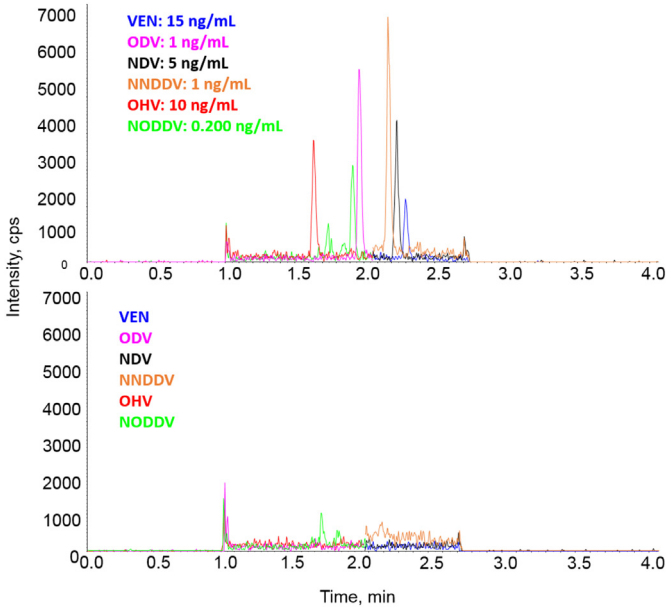

Fig. 3. Top: Representative chromatogram at the lower limit of quantitation (LLOQ) for each analyte: Venlafaxine (VEN), *D,L*-*O*-desmethyl venlafaxine (ODV), rac-*N*-desmethyl venlafaxine (NDV), *D,L*-*N*,*N*-didesmethyl venlafaxine (NNDDV), 4-hydroxy venlafaxine (OHV), and rac-*N*,*O*-didesmethyl venlafaxine (NODDV). Bottom: Representative chromatogram of matrix blank.

3.3.5. Selectivity and matrix effect

The chromatographic regions of the analytes and the internal standards in the 6 lots of blank rat plasma were free from significant interference (≤ 20% of the analyte response at the LLOQ and ≤ 5% of the mean internal standard response of the accepted calibration standards). No significant matrix effects for the analytes or the internal standards were observed in rat plasma. A representative calibration curve of the LLOQ and matrix blank are presented in Fig. 3.

3.3.6. Carryover

The chromatographic regions of the analytes or the internal standards in the matrix blank sample following the ULOQ were free from significant interference (≤ 20% of the analyte response at the LLOQ and ≤ 5% of the mean internal standard response of the accepted calibration standards).

3.3.7. Recovery

The overall recoveries using liquid-liquid extraction were 83.9%, 84.4%, 84.5%, 69.9%, 77.5%, and 74.7% for VEN, ODV, NDV, NNDDV, OHV, and NODDV, respectively. The overall recoveries for the internal standards were 90.4%, 90.1%, 89.2%, 81.3%, 78.2%, and 76.0% for each deuterated internal standard, respectively. Thus, the recovery was adequate to obtain precise and accurate determination in the specified assay range for each analyte.

3.3.8. Hemolysis effect

The results of the hemolyzed samples indicate that hemolysis did not affect assay performance. Precision and accuracy for all analytes ranged from 5.5%–12.8% and 94.0%–110.0%, respectively.

3.3.9. Batch size determination

The maximum number of injections allowed within a single batch was determined to be 96 samples.

3.3.10. Stability

The experimental results showed that the stock standard solution of each analyte was stable for at least 6 h at the RT. The stock standard solution of VEN and NDV was stable for at least 66 days at 4 °C. The stock standard solution of ODV was stable for at least 84 days at 4 °C. The stock standard solution of NNDDV was stable for at least 15 days at 4 °C. The stock standard solution of NODDV was stable for at least 99 days at 4 °C. The stock standard solution of OHV was stable for at least 126 days at 4 °C. The working standard solutions of each analyte were stable at 4 °C for at least 27 days.

Sample collection stability for each analyte was confirmed for up to 2 h on wet ice and at RT. Extracted plasma samples were determined to be stable for up to 24.5 h after processing at 6 °C. Ambient temperature stability for each analyte was confirmed for up to 20 h. All analytes were stable for up to 4 freeze/thaw cycles. Long-term storage stability was established for all analytes for 104 days at −80 °C. The stability of all analytes in plasma is summarized in Table 5.

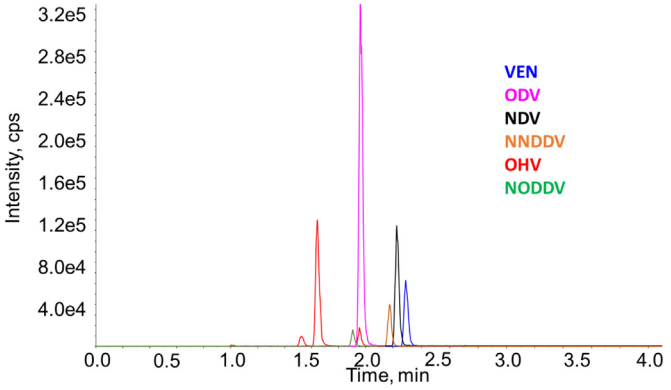

Fig. 4. Representative chromatogram of an incurred rat plasma sample for each analyte: Venlafaxine (VEN), *D,L-O*-desmethyl venlafaxine (ODV), rac-*N*-desmethyl venlafaxine (NDV), *D,L-N,N*-didesmethyl venlafaxine (NNDDV), 4-hydroxy venlafaxine (OHV), and rac-*N,O*-didesmethyl venlafaxine (NODDV).

3.3.11. Reinjection reproducibility

The results indicated that the reinjection reproducibility was acceptable for up to 127 h at 6 °C.

3.4. Pharmacokinetic application

The validated LC-MS/MS method was successfully applied to determine VEN and its five metabolites simultaneously in a PK study, in which 6 healthy rats were orally administrated with VEN at 200 mg/kg. A representative chromatogram of an incurred sample is shown in Fig. 4. The mean plasma concentration *versus* time plots for VEN and its five metabolites are shown in Fig. 5. The PK parameters of analytes such as half-life of drug elimination at the terminal phase ($t_{1/2}$), time of maximum plasma concentration (t_{max}), maximum plasma concentration (C_{max}), area under the plasma concentration-time curve from 0 h to the time of last measurable concentration (AUC_{0-t}), and area under the plasma concentration-time curve from 0 h to infinity ($AUC_{0-\infty}$) were calculated using WinNonlin and data are listed in Table 6.

4. Discussion

The LC-MS/MS method was evaluated over 3 days by measuring QCs at 5 concentrations per analyte. The established LLOQ were 15.0, 1.00, 5.00, 1.00, 10.0, and 0.200 ng/mL for VEN, ODV, NDV, NNDDV, OHV, and NODDV, respectively. The established ULOQ were 6000, 400, 2000, 400, 4000, and 20.0 ng/mL for VEN, ODV, NDV, NNDDV, OHV, and NODDV, respectively. The assay showed excellent linearity ($R \geq 0.996$) for all analytes. Precision and accuracy were evaluated by spiking known concentrations of the compound into blank rat plasma. In 3 consecutive runs, accuracy ranged from 92.0%–105.3% for VEN, 94.0%–103.3% for ODV, 89.3–100.7% for NDV, 86.0%–99.7% for NNDDV, 91.3%–106.0% for OHV, and 94.0%–109.3% for NODDV. Precision over three consecutive runs ranged from 3.2%–10.1% for

Table 5
Confirmation of QC sample stability.

Analyte		Stability of extracted plasma samples at 6 °C for 24.5 h		Stability in plasma for 20 h at room temperature		4 Freeze/thaw cycles at −80 °C/RT		Stability in plasma for 104 days at −80 °C	
		LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
VEN	Accuracy (%)	108.0	99.3	101.1	96.0	107.1	94.2	104.4	103.3
ODV	Accuracy (%)	103.0	102.0	90.0	95.7	86.3	97.7	101.7	104.3
NDV	Accuracy (%)	110.7	104.7	102.7	94.7	103.3	97.3	108.7	106.0
NNDDV	Accuracy (%)	91.7	102.7	86.7	91.7	88.0	92.3	107.0	108.0
OHV	Accuracy (%)	105.3	99.7	88.0	95.0	98.3	98.7	110.0	100.7
NODDV	Accuracy (%)	105.5	112.0	97.5	96.0	103.5	103.3	91.3	92.0

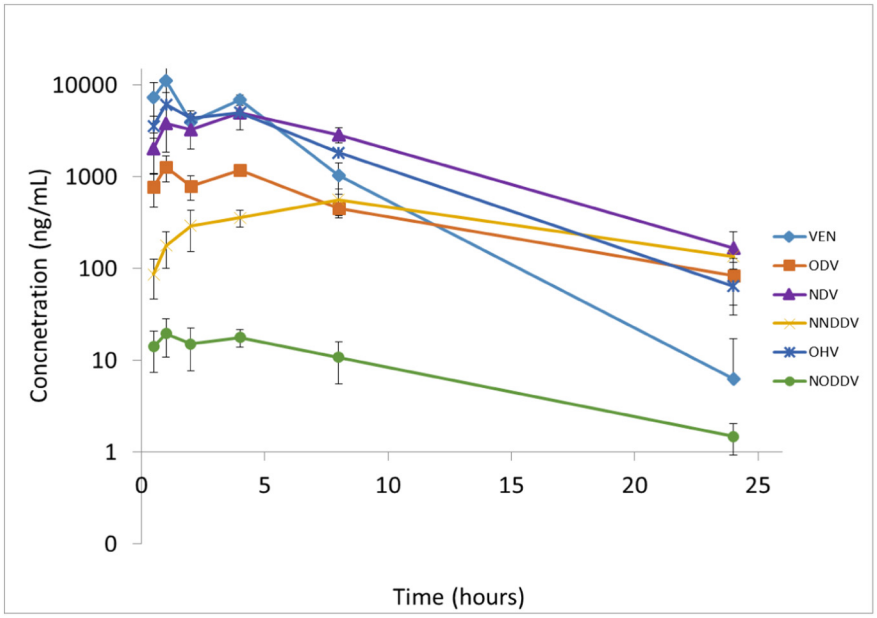

Fig. 5. Concentrations of VEN and its metabolites *versus* time after a single PO 200 mg/kg dose to female rats ($n = 3$).

Table 6
Pharmacokinetic Parameters for VEN and Its Metabolites after a Single 200 mg/kg PO Dose.

Analyte	$t_{1/2}$(h)	t_{max} (h)	C_{max}(ng/mL)	AUC_{last} (h * ng/mL)	AUC_{Inf} (h * ng/mL)
VEN	2.03	1	11,095	49,164	49,182
ODV	5.58	1	1284	11,263	11,943
NDV	4.03	4	4949	53,798	54,772
NNDDV	NR[a]	8	562	8410	10,693
OHV	3.22	1	6132	46,801	47,102
NODDV	5.57	1	20	218	229

[a] NR: Not reportable because $R^2 < 0.85$.

VEN, 2.1%–8.3% for ODV, 2.7%–14.2% for NDV, 2.4%–11.2% for NNDDV, 2.8%–7.8% for OHV, and 4.2%–8.2% for NODDV. The overall recovery of the analytes and the internal standards ranged from 69.9% to 90.4%.

Short-term stability of VEN and metabolites in rat plasma was established for 4 freeze/thaw cycles at −80 °C-to-RT and for at least 20 h at the RT. Long-term storage stability was established for 104 days at −80 °C. Reinjection reproducibility of the extracted samples was demonstrated by reinjecting standards and QCs after storage for 127 h at 6 °C. No matrix effect was observed when comparing the results of QCs prepared in blank matrix with the results of QCs prepared in neat solution.

5. Conclusions

The LC-MS/MS method reported is sensitive, selective, and reliable for simultaneously quantification of VEN and its 5 metabolites (ODV, NDV, NNDDV, OHV and NODDV) in rat plasma. The method was validated by following the US FDA guidance for bioanalytical method validation. The assay quantitation ranges are 15.0 to 6000 ng/mL for VEN, 1.00 to 400 ng/mL for ODV, 5.00 to 2000 ng/mL for NDV, 1.00 to 400 ng/mL for NNDDV, 10.0 to 4000 ng/mL for OHV, and 0.200 to 20.0 ng/mL for NODDV. The validated method was successfully applied to a PK study of VEN orally administered to rats.

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