Tandem Mass Spectrometry (MS/MS) in the Preclinical Pharmacokinetic Study of a Highly Prescribed SNRI Drug for Depression and Its Application to Oral Extended Release Solid Dosage Formulations in Rabbits

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Research Article

Keywords: Preclinical pharmacokinetics, tandem mass spectrometry (MS/MS), venlafaxine, SNRI, extended release, New Zealand White rabbits

Posted Date: December 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1139612/v1

Abstract

Preclinical pharmacokinetic (pK) studies in animal models during the formulation development phase give preliminary evidence and near clear picture of the pK behavior of drug and/or its dosage forms prior to clinical studies on humans and help in tailoring of the dosage form according to the expected and requisite clinical behavior. The present work reports first of its kind preclinical pK study on oral extended release (ER) solid dosage formulations of venlafaxine (VEN) in New Zealand White rabbits. The VEN is a highly prescribed and one of the safest and most effective therapeutic agents used in the treatment different types of depression disorders worldwide. The LC-MS/MS bioanalytical method developed for this purpose demonstrated enough reliability in simultaneously quantitating VEN and its equipotent metabolite *O*-desmethylvenlafaxine (ODV) in rabbit plasma. The method described uses solid phase extraction for sample preparation followed by an ultra-fast LC-MS/MS analysis. The chromatographic separation was achieved isocratically with a predominantly polar mobile phase by employing RPLC. The triple quadrupole LC/MS/MS system operated in MRM mode used an ESI probe as an ion source in positive polarity. The validation results are within the permissible limits of US FDA recommendations and acceptance criteria for bioanalytical method validation.

1. Introduction

The preclinical testing for drug release in oral extended release (ER) solid dosage formulations, which also include tablets and capsules, comprises of *in vitro* dissolution studies and *in vivo* pharmacokinetic (pK) studies in suitable animal models. The animal model preferred should have capacity to house the particular type of ER formulation under preclinical investigation [123]. The data obtained from preclinical pK studies gives preliminary evidence about drug absorption rates and sites, and, possible mechanism of drug distribution, metabolism and elimination. This preclinical pK data collected in animal models also helps in screening prototype ER formulations to support the development and selection of an optimal dosage form for clinical pK studies in humans [45].

Venlafaxine (VEN), (*RS*) 1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol, which belongs to the pharmacological class of serotonin noradrenaline reuptake inhibitors (SNRIs) is a relatively new and structurally novel antidepressant drug [6] was introduced by Wyeth in 1993. It is chemically unrelated to tricyclic, tetracyclic and other antidepressants [78], and, is currently a highly prescribed and one of the most effective drug with fewer unwanted side effects used in the treatment of depression disorders[9]. The VEN is administered orally in both immediate release (IR) and extended release (ER) dosage forms [1011]. The antidepressant action of VEN and its major as well as an equipotent active metabolite, *O*-desmethylvenlafaxine (ODV) [12] (Figure 01) in humans is linked to their potentiation of neurotransmitter activity in the central nervous system. In human plasma, ODV predominates VEN [13] in most of the people except for slow metabolizers, where VEN has been found in higher concentration than ODV [1415]. Both VEN and ODV are potent inhibitors of serotonin and noradrenaline reuptake, and also weakly inhibit the dopamine reuptake [16]. Venlafaxine being a racemate, the *R*-(-) and *S*-(+) enantiomeric forms are present

in equal amounts and both contribute towards its antidepressant activity. The *R*-enantiomer of VEN is potent in inhibiting the synaptosomal serotonin and noradrenaline reuptake, while as the *S*-enantiomer is more selective in inhibiting the serotonin reuptake. However, both the enantiomers of VEN are more potent in inhibiting the serotonin reuptake in contrast to that of noradrenaline [171819]. The enantiomers of ODV also inhibit both noradrenaline and serotonin reuptake with *R*-enantiomer being more potent [2021].

Drug Metabolism Division, Wyeth-Ayerst Research, Princeton, New Jersey reported the initial preclinical pK and metabolic disposition studies of VEN (WY-45,030) including its enantiomers and ODV (WY-45,233) in mouse, rat, dog and rhesus monkey while VEN and ODV were still under development. The analysis of plasma and urine obtained after the animals received pure aqueous solution of VEN and ODV, and not any kind of dosage formulation, intravenously (*i.v.*) and/or intragastrically (*i.g.*) was conducted by high performance liquid chromatography (HPLC) method with UV detection. The Wyeth-Ayerst Research also reported an HPLC method for simultaneous estimation of VEN and ODV in rat, dog and mouse plasma [18222324]. In last 30 years, after its approval for the clinical use, a sizeable number of bioanalytical methods for the quantification of VEN and ODV in human plasma and urine have been reported in literature, but the same does not hold true for preclinical animal models. After a comprehensive review of available literature till date, the following bioanalytical methods for carrying out preclinical pK studies of VEN and ODV in animals were found and almost all of these reports administering aqueous solutions of VEN and ODV in its pure form through an oral, *i.v.* or *i.g.* route. da Fonseca and Bonato (2010) reported an *in vitro* enantioselective biotransformation study of VEN to its metabolites in liver microsomal preparations of male Wistar rats using chiral HPLC separation with UV detection [25]. Zhang et al. (2017) developed HPLC method with mass spectrometric detection (LC-MS) for the *pK* studies of VEN solid dispersions in male Wistar rats [26]. The tandem mass spectrometry (MS/MS), having higher specificity and improvements like high signal-to-noise ratio, accuracy and reproducibility, coupled with HPLC has become an apt and more effective tool for extremely low detection limits and is considerably applied to pK studies at present [27]. Many papers have reported HPLC and ultra-high performance liquid chromatography (UHPLC/UPLC) tandem mass spectrometric methods (LC-MS/MS) for the quantification of VEN and ODV in rat plasma. Shah et al. (2009) and Ahmad et al. (2016) have reported liquid-liquid extraction (LLE) and solid-phase extraction (SPE) based LC-MS/MS methods respectively for the simultaneous estimation of VEN and ODV in rat plasma [2829]. Aryal *et al.* (2011) has successfully utilized an LC-MS/MS method to investigate the pK parameters of VEN and ODV in mice plasma and brain dialysate after an *i.v.* and *i.g* administration of the drug [30]. da Fonseca and Bonato (2013) reported since in their previous work [25], the quantification limit attained by HPLC-UV method was appropriate for *in vitro* biotransformation studies only, therefore for assessing the kinetic disposition of VEN, ODV and *N*-desmethylvenlafaxine (NDV) in rat plasma after the oral administration of VEN, a more selective and sensitive technique like LC-MS/MS method was developed [31]. An LC-MS/MS method was used by Zhang et al. (2013) to determine the concentration of phenolic esters of ODV, and, Liu et al. (2016) to estimate synthetic prodrugs of ODV in plasma, brain and hypothalamus of male Wistar rats, along with pK parameters in beagle dogs in both experimentations after administering the aqueous drug

solutions *i.g.* and orally to animals [3233]. The validation of a UPLC-MS/ESI method developed for simultaneous determination of VEN and ODV in rat plasma and its use in pK studies in male Wistar rats, after administering the VEN solution orally, has been carried out by Dubey et al. (2013) [34]. Pan et al. (2016) claimed to have developed a fully validated UPLC-MS/MS method for simultaneous estimation of methadone, fluoxetine, VEN and their metabolites in spiked rat plasma for drug interaction study and additionally applied it to pK studies as well [35]. An effective UHPLC-MS/MS method for the simultaneous quantification of VEN and its five metabolites in rat plasma have been reported by Gu et al. (2018) and simultaneously applied it for pK study of VEN orally administered to rats [36]. Chen et al. (2020) has reported a UPLC-MS/MS method to investigate the underlying mechanism of effect of vonoprazan on VEN in rat liver microsomes along with its impact on pK profile of VEN and ODV in male Sprague-Dawley rats after oral administration of drugs [37]. An SPE based gas chromatography mass spectrometric (GC-MS) method for the estimation of venlafaxine in rat plasma and its application to assess pK interaction between VEN and fluoxetine in Sprague-Dawley rats has been reported by Song et al. (2017) [38]. One spectrofluorometric method for the estimation of VEN in spiked rat plasma reported by Shahnawaz et al. (2010) was also found in literature [39].

Nerkar and Gattani (2012, 2013) in two separate studies have reported a comparative pK profile of *i.v.*, oral solution and buccal mucoadhesive gel formulations (Cress seed and Linseed based) containing VEN in New Zealand white rabbits using an HPLC-UV method [4041]. In an another pK study reported by Peng et al. (2013), an HPLC method has been employed for the determination of VEN in rabbits after injecting a VEN saline solution and thermosensitive VEN-chitosan hydrogel subcutaneously [42]. Ali et al. (2019) has also employed an HPLC-UV method for pK studies of VEN containing oral solution and sustained release hydrogel composites in rabbits [43]. An HPLC-UV method developed and validated in rabbit plasma by Sher et al. (2020) has been applied to pK analysis of VEN in albino rabbits after its oral administration [44].

After reviewing the available literature thoroughly, only two reports of preclinical pK studies of a prototype oral ER solid dosage form containing VEN conducted in any animal model were found. The first one is of an VEN ER tablet reported in Rowley et al. (2005) United States patent US 20050136109A1 by Wyeth. The patent mentions the pK studies of VEN ER tablets conducted in Beagle dogs, however, no account of bioanalytical method utilized for estimation of VEN and ODV from dog plasma is stated in the patent publication [45]. The other one is an *in vivo* pK study of chitosan-carbomer matrix tablets containing VEN conducted by Zhang et al. (2018) in Beagle dogs employing an HPLC method [46].

The present study, to the best of our knowledge, is the first report on preclinical pK studies of oral ER solid dosage formulations of venlafaxine in New Zealand White rabbits. The study involves bioequivalence (BE) assessment of an in-house produced venlafaxine HCl ER matrix tablet and Efexor XR capsule (US FDA reference listed drug) employing SPE based robust, reliable and validated ultra-high performance LC-MS/MS method for simultaneous quantitation of VEN and ODV in rabbit plasma.

2. Methods

2.1. Chemicals and materials

Working standards of venlafaxine hydrochloride and *O*-desmethylvenlafaxine of 99.83% and 99.82% purity respectively were purchased from Vivan Life Sciences (India). Deuterium labeled venlafaxine-D6 hydrochloride (VEN-D6) of 99.46 atom% D-isotropic enrichment & 99.68% purity and Rac-*O*-desmethylvenlafaxine-D6 succinate hydrate (ODV-D6) of 99.92 atom% D-isotropic enrichment & 99.02% purity was also procured from Vivan Life Sciences (India) and used as an internal standard (IS). Reagent grade ammonium formate and HPLC grade acetonitrile and methanol were obtained from Honeywell Specialty Chemicals (GmbH). Reagent grade orthophosphoric acid and liquor ammonia were acquired from Thermo Fisher Scientific (India). Reagent grade formic acid was obtained from Sigma-Aldrich Chemicals (India). All aqueous solutions and buffers were prepared using ultrapure Milli-Q® water. SPE cartridges (Bond *Elut* PLEXA, 30mg/1cc) were obtained from Agilent (USA). Sodium heparin containing blood collection tubes, BD Vacutainer®, were purchased from Becton Dickinson (USA). Efexor XR 37.5 mg was purchased from Pfizer Ireland Pharmaceuticals (Ireland).

2.2. Preparation of calibration standards and quality controls

Stock solutions (1 mg mL^{-1}) of VEN and ODV were prepared by dissolving accurately weighed standard compounds in methanol. Working solution in the range of 10 to 5000 ng mL^{-1} was prepared by diluting the stock solution with diluent (50% methanol in water, v:v). The calibration standard (CS) and quality control (QC) samples were prepared from working solution using diluent and spike-in rabbit plasma (%spiking ~ 5%). The calibration curve (CC) ranged from approximately 0.5 to 250.0 ng mL^{-1} for both analytes. QC samples were prepared at limit of quantitation (LOQQC), low (LQC), medium (M1QC, MQC) and high (HQC) concentration levels. The nominal concentration of CS and QC samples prepared in rabbit plasma is given in Table 01.

Table 01
Nominal concentration of calibration standard and quality control samples

Samples	ID	Nominal conc. (ng mL^{-1})	
		VEN	**ODV**
CSs	STD-H	0.503	0.493
	STD-G	1.378	1.350
	STD-F	6.891	6.749
	STD-E	19.689	19.283
	STD-D	49.222	48.208
	STD-C	98.444	96.416
	STD-B	196.889	192.833
	STD-A	255.700	250.432
QCs	LOQQC	0.504	0.494
	LQC	1.392	1.363
	M1QC	49.222	48.208
	MQC	98.444	96.419
	HQC	196.889	192.883

2.3. Sample preparation

For sample preparation, SPE method was developed for the extraction of VEN and ODV from rabbit plasma. Frozen samples were allowed to thaw at room temperature before vortex-mixing to homogenize the contents. To an 80 µL aliquot of rabbit plasma in a polypropylene tube, 50 µL of IS dilution (containing ~ 25.0 ng mL^{-1} of VEN-D6 and ODV-D6) followed by 400 µL of pretreatment solution (10% orthophosphoric acid in water, v:v) was added and vortexed. The samples were transferred to SPE cartridges (preconditioned with 0.5 mL of methanol followed by 0.5 mL of Milli-Q® water and centrifuged at 2000 rcf for a minute after each addition) and centrifuged at the rcf same as that of preconditioning for one minute. The cartridges were washed with 1 mL of washing solution (5% liquor ammonia in water, v:v) and Milli-Q® water by running centrifuge at 2000 rcf for a minute after each addition. The samples were eluted with 1 mL of methanol by centrifuging at 2000 rcf for a minute and dried under nitrogen steam at 50±4°C and at about 20±5 psi. The dried sample residues were reconstituted in 300 µL of mobile phase, vortexed and an aliquot of the resulted sample was injected onto the LC-MS/MS system for analysis.

2.4. Liquid chromatography (LC)

A shimadzu Nexera X2 UHPLC system consisting of two Nexera X2 LC-30AD pumps, a Nexera X2 SIL-30AC MP auto sampler, an online DGU-20ASR degassing unit and a CTO-20AC Prominence HPLC column oven was used for LC. Chromatographic separation was achieved *via* isocratic elution of mobile phase (60% methanol in 2mM ammonium formate buffer (v:v) + 0.1% formic acid L^{-1}) at a flow rate of 0.300 mL min^{-1} on an ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 µm) with a total run time of 3 minutes. An injection volume of 5 µL was used for each analysis. The retention window for VEN and VEN-D6 was 1.1 to 1.4 min and 0.75 to 1.1 min for ODV and ODV-D6. The auto sampler and column oven temperature were set at 10±1.0°C and 40±1.0°C respectively. The composition of rinsing solution used was 90% acetonitrile in water (v:v).

2.5. Tandem mass spectrometry (MS/MS)

The eluted samples from LC were subsequently analyzed using an AB SCIEX triple quadrupole API 4000™ LC/MS/MS system operated in multiple reaction monitoring (MRM) mode. The ion source used was TurboIonSpray®, an electrospray ionization (ESI) probe, in positive polarity. Two MRM transitions, Q1 (precursor ion) and Q3 (product ion), with a dwell time of 200 ms for simultaneous quantitation and identification of the analytes (VEN, ODV) and internal standards (VEN-D6, ODV-D6) based on mass-to-charge ratio (m/z) calculation and compound dependent parameters were optimized.

2.6. Data processing and statistical evaluation

LC/MS/MS data was processed using the Analyst® software version 1.6.3 from AB SCIEX. The CC was generated by means of a weighted (X^{-1}, X^{-2} and none, where X = concentration) linear regression and values for slope, intercept and correlation coefficient (R) were obtained. The concentration of VEN and ODV was determined by plotting peak area ratio of analyte/IS based on CC. Mean, standard deviation (SD), precision (%CV), accuracy (%Nominal) were calculated using MS Excel software.

2.7. Validation

The method developed was validated in line with US FDA Bioanalytical Method Validation Guidance for Industry 2018 [47]. The validation was done with regard to linearity of CC, sensitivity at LOQ, selectivity, carry over, precision and accuracy (PA) and recovery as per the FDA recommendations and acceptance criteria for bioanalytical method validation.

2.8. Preclinical pK studies of VEN and ODV in rabbits

The bioanalytical method developed was used for simultaneous quantitation of VEN and ODV and applied for carrying out preclinical pK studies of VEN and ODV in rabbit plasma. The preclinical pK studies in New Zealand White rabbits was approved by the statutory Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Government of India through Institutional Animal Ethics Committee (IAEC) of Department of Pharmaceutical Sciences, University of Kashmir (Ref. No. F[IAEC-Approval]KU/2017/09). The animal experiments were conducted in accordance with the relevant Indian Laws, The Breeding and Experiments on Animals (Control and Supervision) Rules 1998, Amendment Rules 2001, Amendment Rules 2006, and the ARRIVE guidelines. Four pairs

(male/female) of healthy rabbits, each weighing 3 – 4 Kg, were provided by Government Rabbit Farm, Wussan (Pattan), Kashmir. The rabbits were divided into a reference group and a test group. Each of the 4 rabbits in reference group received a capsule of Efexor XR 37.5 mg orally and in the same manner rabbits in test group received an in-house formulated 37.5 mg venlafaxine HCl ER matrix tablet. After administration, 2 mL of blood was collected from marginal ear vein at 0, 1, 2, 3, 4, 6, 8, 12, 24, and 36 hours in sodium heparin containing BD Vacutainer®. The cell free plasma from anticoagulated blood was obtained by centrifuging the blood samples at 3000 rcf and 17°C for 15 minutes as per the WHO Use of Anticoagulants in Diagnostic Laboratory Investigations 2002 guidelines [48]. The resulting plasma samples were frozen at –40°C until further analysis.

3. Results And Discussion

3.1. Method development

During method development SPE was found to be more selective for removing the interferences like dissolved salts (electrolytes) and proteins from the plasma. The reliability of the method was ascertained from the results of recovery experiment delivering excellent reproducibility. The requirement of 80 µL aliquot of rabbit plasma also ensured less wasted sample. A non-ionic and non-polar base load method for primary extraction of analytes having Log P > 1.5 and pK_a of 6 – 10 was selected, and SPE cartridges containing hydrophilic non-polar styrene divinylbenzene polymeric sorbent were used for this purpose. The sorbent conditioning was achieved with 100% methanol followed by equilibrating with 100% water. The acidic treatment of plasma with orthophosphoric acid helped in protein precipitation and made plasma ready for extraction loading. During loading the gradient of polarity on the hydrophilic sorbent surface allowed phase transfer of analytes into the more hydrophobic polymer core for retention prior to washing and subsequent elution. The large endogenous matrix components did not reach to the core due to their inability to bind to the polymeric surface. The washing treatment with liquor ammonia removed the interferences without leaching of analytes. The clean extract with high recovery was obtained by eluting the plasma sample with 100% methanol.

The focus during LC was on obtaining sharp peaks with high resolution for the analytes under investigation and attaining high efficiency coupled with maximum MS/MS sensitivity. The chromatographic separation was realized isocratically using reversed-phase LC (RPLC). According to the fundamental resolution equation for isocratic separations, resolution depends on column efficiency, which in itself is influenced by the particle size of the stationary phase. Since the smaller particles (sub-2-µm) provide higher chromatographic resolution, UPLC BEH C_{18} column was selected for RPLC. The stationary bonded phase of this column contains highly efficient 1.7 µM ethylene bridged ethyl-siloxane/silica hybrid (BEH) particles bonded with hydrophobic trifunctional C_{18} ligands utilizing proprietary endcapping processes. The bonded phase has a ligand density of 3.1 µMol M^{-2} and carbon load of 18%. Since retention in RPLC is primarily related to solute hydrophobicity, for the molecule more hydrophobic to be separated, the lesser hydrophobic ligand ought to be used. VEN and ODV being highly

hydrophilic molecules, strongly hydrophobic trifunctional C_{18} ligands were able to provide sufficient binding for desired separation. The appropriate adjustment of polarity of the mobile phase is equally important as the selection of the non-polar column for the separation of solute molecules in RPLC. The predominantly polar solvent methanol was optimized as the mobile phase and the adequate buffering capacity of the mobile phase was maintained with ammonium formate (pK_a = 3.75 and buffer range of 3.76 – 5.76). Since the flow rate of mobile phase plays an important role in the resolution of small molecules in reversed phase separations, attuning it to 0.300 mL/min helped in obtaining high resolution for the analytes.

The two main targets taken into consideration while optimizing MS/MS conditions were achieving adequate sensitivity and selectivity. Although sample extraction, chromatography development and MS initial tuning all have an effect on sensitivity and selectivity, MS optimization considerably influences the sensitivity of the method. During the MS initial tuning the first step was determining the ionization of analytes. The ionization source/gas parameters (Table 02) such as gas flow and ionization voltage were tuned to the analytes making optimum ionization conditions for the analyte and subsequently sensitivity. It was during this MS initial tuning, the positive ionization mode was determined for carrying out MS/MS analysis. The appropriate ionization mode and detection polarity was selected on the basis of analyte polarity and LC operating conditions. The major improvement the use of ESI offers is formation of protonated or deprotonated molecules with little fragmentation, which is ideal for selection of precursor ions including maximizing sensitivity.

Table 02: Parameter optimization for electrospray ionization (ESI) probe

Parameter	Values
Gas 1 – IonSpray nebulizer gas	60 psig
Gas 2 – TurboIonSpray® heater gas	40 psig
Temperature	500 °C
Curtain gas	30 psig
IonSpray voltage	5500 V
Collision gas	6 psig
Interface heater	ON

The tandem mass spectrometry is principally based upon precursor ion selection, its fragmentation mostly by collision-induced dissociation (CID) and the m/z measurement of the product ions formed. The screening of two to three MRM transitions for each analyte in the beginning gives the confidence that the correct analyte is being monitored and this selectivity helps in the later stages of method development when actual biological samples are analyzed. The optimized MS parameters for each compound are given in Table 03. The analytes were detected by collision induced MS/MS employing MRM and mass

transition ion-pair (precursor-product ion transitions) for VEN, ODV, VEN-D6 and ODV-D6 was selected as m/z 278.2→58.1, m/z 264.0→58.1, m/z 284.2→64.1, m/z 270.0→64.1 respectively.

Table 03: Optimized MS parameters for MRM transitions of each compound

Compound	MRM		Compound parameters			
	Q1 (amu)	Q3 (amu)	DP (V)	EP (V)	CE (V)	CXP (V)
VEN	278.2	58.1	40	10	35	6
ODV	264.0	58.1	40	10	35	6
VEN-D6	284.2	64.1	40	10	35	6
ODV-D6	270.0	64.1	40	10	35	6

3.2. Method validation

3.2.1. Linearity of CC

All blank, CS and QC samples were prepared in the plasma obtained from the same rabbits as the study samples. Three batches of spiked plasma samples containing eight non-zero CS levels including an LOQ and covering the quantitation range were run for linearity of CC. Every run also included 2 replicates of each LQC, M1QC, MQC and HQC. All non-zero CS levels were found to be ±15% of the nominal concentrations as per the FDA acceptance criteria. The concentration-response relationship was found to be fit with simplest regression model and showed linear character of CC. The average CC parameters for each analyte are presented in Table 04.

Table 04: Average *CC* parameters for VEN and ODV in rabbit plasma

Average calibration curve parameters				
Analyte	**CC run**	**Slope**	**Intercept**	**R**
VEN	I	0.0427	-0.000968	0.9995
	II	0.0433	-0.00114	0.9996
	III	0.0422	0.00068	0.9997
ODV	I	0.0392	0.00437	0.9995
	II	0.0407	0.00431	0.9991
	III	0.0385	0.00376	0.9994

3.2.2. Sensitivity at LOQ

The lowest non-zero CS level on the CC defines the LOQ. FDA acceptance criteria requires LOQ to be ≥ 5 times the analyte response of the zero CS level (blank), with an accuracy of ±20% of the nominal concentration and reproducible with a precision of ±20% (%CV). Sensitivity at LOQ was done as a part of PA assessment for the CC range and was determined by running the LOQQC (Figure 02) in 6 replicates in three within-day PA batches. The results given in Table 05 show that LOQ was quantitatively determined within the acceptable precision and accuracy criteria.

Table 05: Sensitivity at LOQ for each analyte in rabbit plasma

Sensitivity at LOQ				
Analyte	**LOQQC run (Within-day)**	**SD**	**Precision (%CV)**	**Accuracy (%Nominal)**
VEN	PA batch – I	0.10805	19.15	111.97
	PA batch – II	0.10248	18.29	111.14
	PA batch – III	0.02192	4.70	92.56
ODV	PA batch – I	0.07833	15.30	103.61
	PA batch – II	0.08118	16.38	100.30
	PA batch – III	0.02701	6.13	89.24

3.2.3. Selectivity

FDA guidelines ask for carrying out selectivity during validation to lessen or avoid interference from potential interfering endogenous components of matrix and verify the substance being measured is the intended analyte. Selectivity was demonstrated by analyzing 10 blank samples of rabbit plasma (double blank), and, the peak area response at retention times of analytes (VEN and ODV) and ISs (VEN-D6 and ODV-D6) in both blank matrix and extracted LOQ was measured (Figure 03). The extraction method used was same as mentioned in sample preparation section 2.3. The %area of blank matrix to mean peak area response of analyte in extracted LOQ for VEN and ODV was 1.25 to 4.22% and 1.07 to 9.22% respectively. For both ISs, VEN-D6 and ODV-D6, the %area of blank matrix to mean peak area response of IS in extracted LOQ was 0.00 to 0.02%. The results were well within FDA acceptance criteria and showed blanks to be free of interference for the analyte (< 20% peak area response of analyte in blank compared to extracted LOQ samples) and the IS response in blank didn't exceed 5% of the average IS responses of CSs and QCs.

3.2.4. Carryover

In an analytical method, carryover between the samples can occur by means of influx of an analyte in a sample from the preceding one. FDA guidelines demand carryover should not exceed 20% of LOQ and urge riddance of any carryover as well as monitoring its impact, if any, on the quantitation of study samples. Carryover was monitored by injecting 3 blank matrix samples (double blank) subsequently after

the high CS levels. The %area of 3 blank matrix samples to mean peak area response of analyte in extracted LOQ for VEN and ODV was 5.17%, 3.11%, 2.43% and 2.72%, 2.26%, 5.98% respectively. Similarly, for VEN-D6 and ODV-D6, the %area of blank matrix to mean peak area response of IS in extracted LOQ remained in the range of 0.00 to 0.02%. The results exhibit negligible carryover with no enhancement in the peak area response at retention times of analytes and ISs ensuring the carryover didn't affect the PA of the method developed.

3.2.5. Precision and accuracy

Method validation by means of weighing PA is essential as per the FDA guidelines to establish the method qualification for analysis of study samples and involves evaluating 4 *QC* levels (LOQQC*,* LQC, MQC and HQC) across the quantitation range. Between batch (*inter-day*) and within batch (*intra-day*) PA was determined by running VEN and ODV QC replicates against the CC on different days (n=18) and same day (n=6) respectively. Freshly prepared CSs and QCs were used for each run. A minimum of 3 independent PA runs were carried out along with a CC included in each run. Based on the performance of QCs in all between batch and within batch PA runs, it is evident from the results given in Table 06 that PA of the method is well within the FDA acceptance criteria (precision and accuracy: ±15% of nominal concentrations of QCs except for ±20% at LOQQC).

Table 06: Between batch and within batch *PA* results for four *QC* levels of each analyte in rabbit plasma

Analyte	VEN				ODV			
QC levels	LOQQC	LQC	MQC	HQC	LOQQC	LQC	MQC	HQC
Nominal conc. (ng mL^{-1})	0.504	1.392	98.444	196.889	0.494	1.363	96.416	192.833
Between batch PA								
Mean conc. (ng mL^{-1}, n=18)	0.530	1.368	105.538	208.041	0.482	1.332	102.478	206.946
SD	0.009	0.090	8.974	5.397	0.070	0.096	5.633	6.929
Precision (%CV)	17.71	6.64	8.50	2.59	14.55	7.23	5.50	3.35
Accuracy (%Nominal)	105.22	98.32	107.21	105.66	97.72	97.77	106.29	107.32
Within batch PA – I								
Mean conc. (ng mL^{-1}, n=6)	0.564	1.370	105.921	208.395	0.511	1.317	102.381	207.529
SD	0.108	0.111	11.781	6.595	0.078	0.114	7.061	9.499
Precision (%CV)	19.15	8.12	11.12	3.16	15.30	8.66	6.90	4.58
Accuracy (%Nominal)	111.97	98.43	107.60	105.84	103.61	96.66	106.19	107.62
Within batch PA – II								
Mean conc. (ng mL^{-1}, n=6)	0.560	1.369	106.177	208.930	0.495	1.311	102.391	204.891
SD	0.102	0.106	10.380	5.524	0.081	0.106	6.515	6.538
Precision (%CV)	18.29	7.75	9.78	2.64	16.38	8.15	6.36	3.19
Accuracy (%Nominal)	111.14	98.41	107.86	106.12	100.30	96.20	106.20	106.25
Within batch PA – III								
Mean conc. (ng mL^{-1}, n=6)	0.466	1.365	104.516	206.798	0.440	1.369	102.661	208.420
SD	0.021	0.066	5.034	4.701	0.270	0.068	3.940	4.710

Precision (%CV)	4.70	4.84	4.82	2.27	6.13	4.99	3.84	2.26
Accuracy (%Nominal)	92.56	98.12	106.17	105.03	89.24	100.44	106.48	108.08

3.2.6. Recovery

The efficiency and reproducibility of the extraction process used during method development is established by optimizing the recovery of analyte and IS. Although FDA guidelines state that recovery need not to be 100%, but maintain that the extent of recovery be consistent and reproducible. The recovery was performed by comparing the analyte peak area (counts) of 6 replicates each of extracted QC samples at LQC, MQC and HQC with the corresponding extracts of blanks spiked with the analyte post-extraction (representing approximately 100% recovery). The recovery of ISs was estimated in the similar manner using 18 replicates of extracted IS samples. The results of the recovery experiment given in Table 07 confirm the consistency and reproducibility of SPE used in the method development for the simultaneous estimation of VEN, ODV and their ISs.

Table 07: SPE based recovery of VEN, ODV, VEN-D6 and ODV-D6

Analyte	% Recovery			Mean	SD	Precision (%CV)
	LQC	MQC	HQC			
VEN (n=6/QC)	86.41	89.10	88.05	87.85	1.355	1.54
ODV (n=6/QC)	72.42	75.91	73.77	74.03	1.760	2.38
IS	**% Recovery**					
VEN-D6 (n=18)	81.52					
ODV-D6 (n=18)	68.64					

3.3. Pharmacokinetic application of method in rabbits

The validated LC-MS/MS method was efficiently applied to determine simultaneously VEN and ODV in rabbit plasma after oral administration of the reference and test product as described under heading 2.8. The pK parameters such as maximum plasma concentration (C_{max}), time of maximum plasma concentration (t_{max}), elimination half-life of the drug at the terminal phase ($t_{1/2}$), area under the plasma concentration time curve from zero hour to last finite concentration (AUC_{0-36}), area under the plasma concentration time curve from zero hour to infinity ($AUC_{0-\infty}$) and elimination rate constant (K_{el}) analyzed by a non-compartmental model using PKPlus 2.0 software from Simulations Plus, USA are summarized in Table 08. The mean plasma concentration *versus* time plots of VEN and ODV is shown in Figure 04. The plasma concentrations of VEN and ODV remained in standard quantitation range and above LOQ (0.5 ng mL^{-1}) throughout the sampling period (36 h). The BE assessment by means of bioavailability

comparison between reference and test product through key pK parameters (C_{max} and AUC) was found to be similar. This statistical assurance of bioequivalent similarity was weighed using quantitative comparison of the standard error (SE) of the mean, T/R ratio and 90% confidence interval values of C_{max}, AUC_{0-36} and $AUC_{0-\infty}$ of reference and test product as given in Table 09. The representative chromatograms of VEN and ODV C_{max} of reference and test products in given in Figure 05.

Table 08: pK parameters of VEN and free ODV in New Zealand white rabbits (n=4)

Formulation	VEN pK parameters					
	C_{max} (ng mL^{-1})	t_{max} (h)	$t_{1/2}$ (h)	AUC_{0-36} (ng.h mL^{-1})	$AUC_{0-\infty}$ (ng.h mL^{-1})	K_{el} (h^{-1})
Reference	57.69±13.20	3	8.4±0.5	566.21±99.23	596.38±107.16	0.082±0.005
Test	58.19±10.76	4	8.8±0.3	593.17±110.06	628.83±116.16	0.079±0.003
	ODV pK parameters					
Reference	9.55±0.75	3	10.2±1.2	116.50±11.63	126.78±15.29	0.068±0.008
Test	10.12±1.84	3	11.2±0.5	128.66±29.92	143.43±34.93	0.062±0.003

Table 09: Statistical assurance of bioequivalent similarity of reference and test product

Analyte	VEN			ODV		
pK parameters	C_{max} (ng mL^{-1})	AUC_{0-36} (ng.h mL^{-1})	$AUC_{0-\infty}$ (ng.h mL^{-1})	C_{max} (ng mL^{-1})	AUC_{0-36} (ng.h mL^{-1})	$AUC_{0-\infty}$ (ng.h mL^{-1})
% CV (Reference)	22.89	17.53	17.97	7.88	9.99	12.06
% CV (Test)	18.50	18.55	18.47	18.21	23.26	24.35
SE	8.52	74.09	79.02	1.00	16.05	19.06
T/R ratio	100.87	104.76	105.44	105.99	110.44	113.13
Lower 90% CI (T/R)	69.39	76.86	77.19	83.76	81.07	81.07
Upper 90% CI (T/R)	132.35	132.66	133.69	128.22	139.82	145.19

3. Conclusion

The preclinical pK data obtained from small studies, with a limited number of animals and plasma samples, during formulation development phase can offer critical pK information of a drug and its dosage form for attaining the requisite clinical pK behaviour. In this work, we are reporting preclinical BE assessment studies of two ER formulations of VEN, Efexor XR and an in-house formulated ER matrix tablet, in New Zealand White rabbits. For this purpose, a reliable and rapid SPE based LC-MS/MS bioanalytical method, for the simultaneous quantitation of VEN and ODV, was developed and effectively validated in agreement with the permissible limits of FDA recommendations and acceptance criteria.

Declarations

Acknowledgement

1. The authors are thankful to Sun Pharmaceuticals Industries Limited India (Clinical Pharmacology & Pharmacokinetics), for letting to carry out this work at its research and development centre in Gurugram, Haryana.

2. The authors are also grateful to Prof. F.A. Masoodi and Dr. Adil Gani for making the facility for animal study available at Department of Food Sciences & Technology, University of Kashmir, Hazratbal, Srinagar, Kashmir.

Notes

The authors declare no competing financial interest.

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Figures

Figure 1

Chemical structure depiction of [a] venlafaxine and [b] *o*-desmethylvenlafaxine

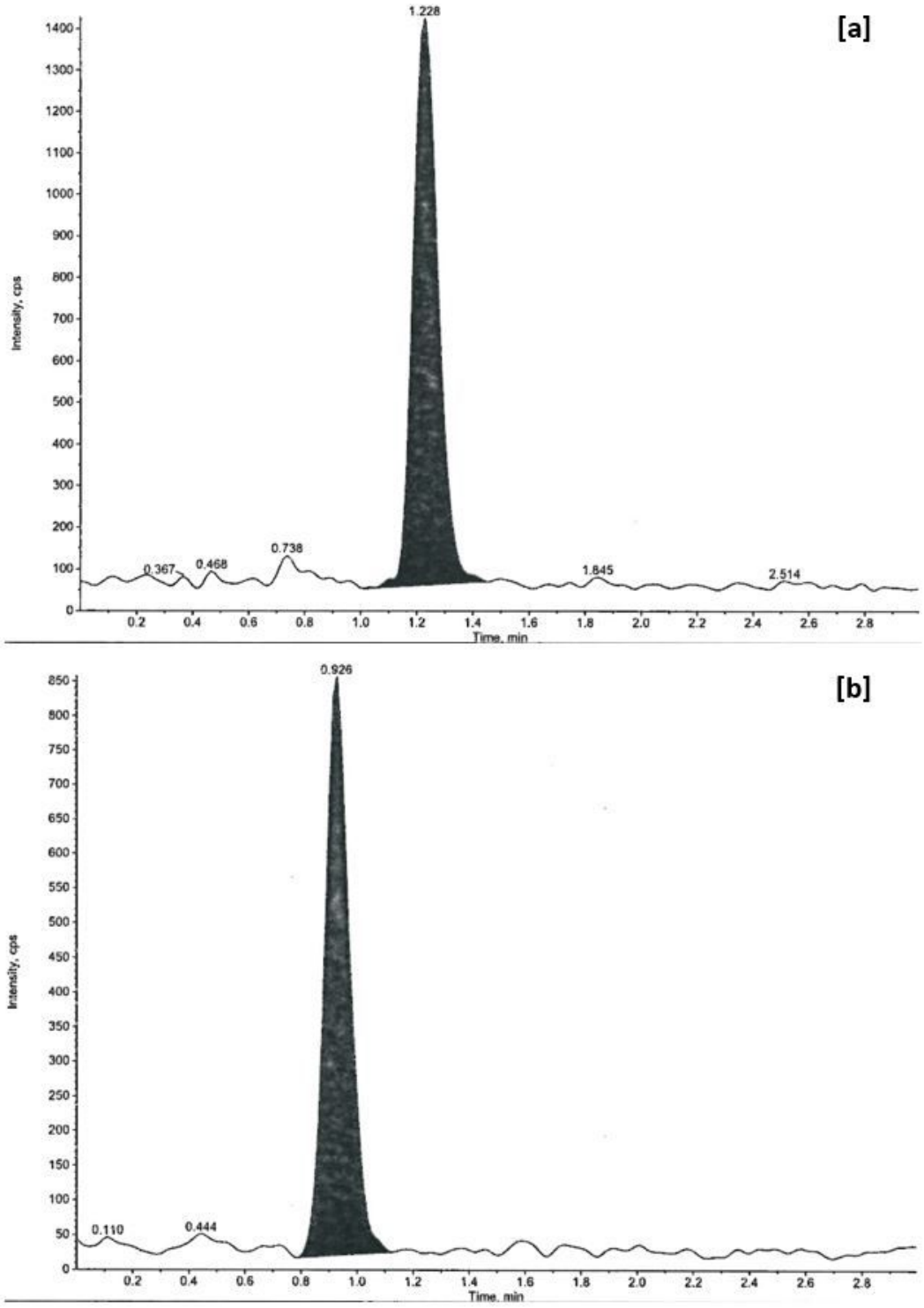

Figure 2

Representative chromatogram of LOQQC of [a] venlafaxine and [b] *o*-desmethylvenlafaxine

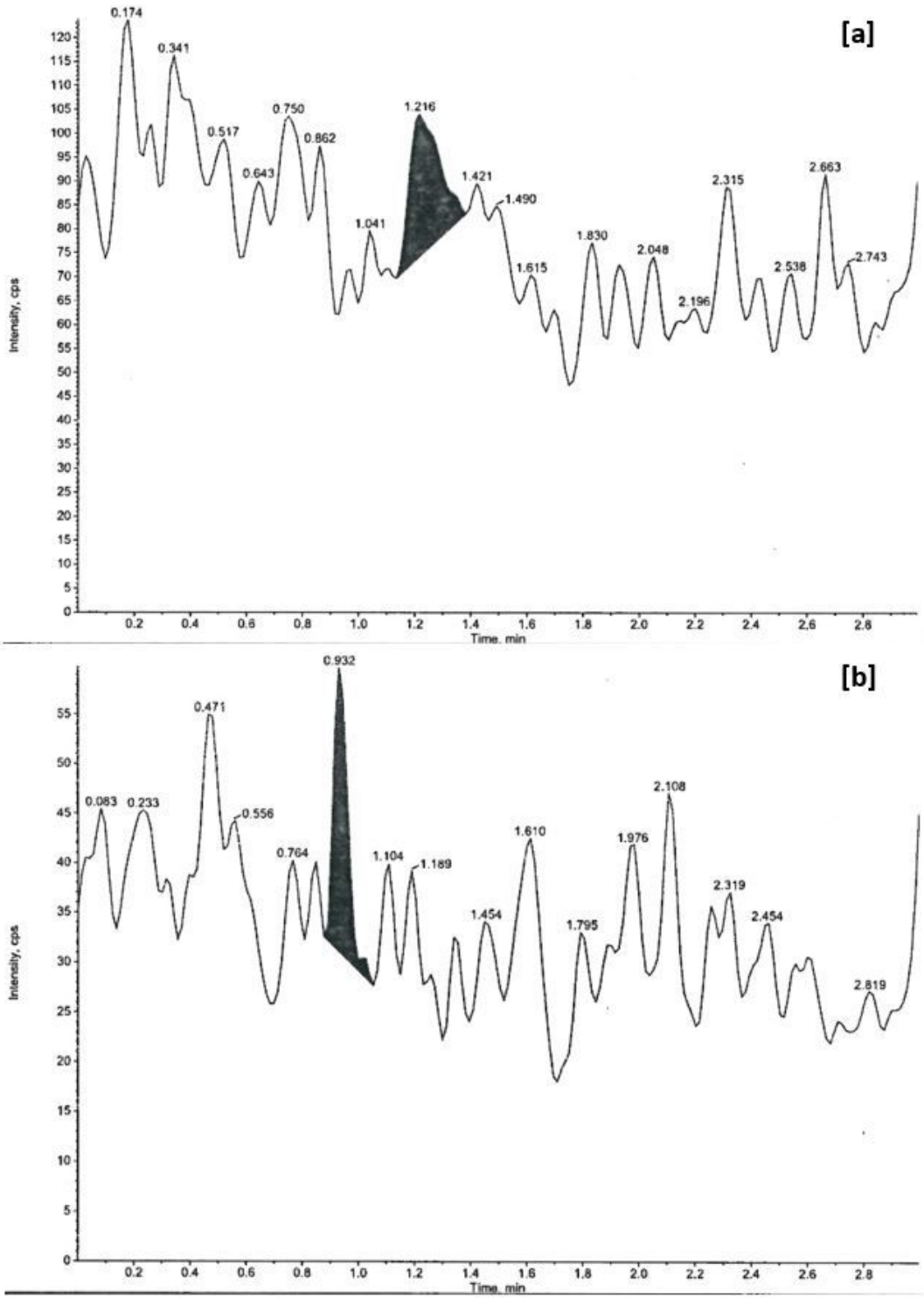

Figure 3

Representative chromatogram of double blank of [a] venlafaxine and [b] *o*-desmethylvenlafaxine

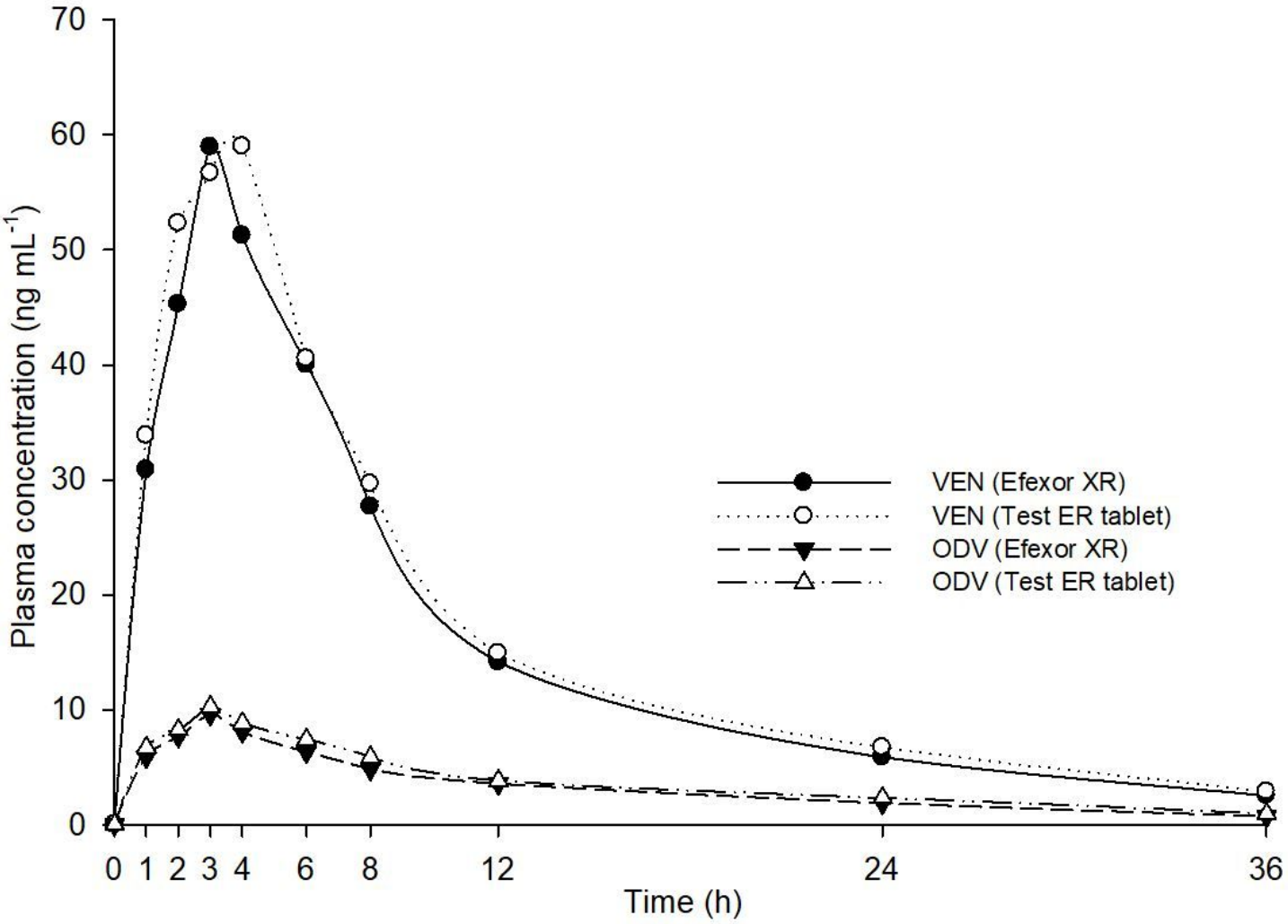

Figure 4

Mean plasma concentration *versus* time plots after single oral dose of Efexor XR 37.5 mg and an in-house formulated 37.5 mg venlafaxine ER tablet in New Zealand white rabbits

Figure 5

Representative chromatograms of [a] venlafaxine C_{max} Ref, [b] venlafaxine C_{max} Test, [c] *o*-desmethylvenlafaxine C_{max} Ref and [d] *o*-desmethylvenlafaxine C_{max} Testa

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