Journal of Chromatography B, 878 (2010) 689–694

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short Communication

Simultaneous quantification of venlafaxine and *O*-desmethylvenlafaxine in human plasma by ultra performance liquid chromatography–tandem mass spectrometry and its application in a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 25 October 2009
Accepted 11 January 2010
Available online 18 January 2010

Keywords:
Venlafaxine
O-desmethylvenlafaxine
UPLC-MS/MS
Human plasma
Pharmacokinetics

ABSTRACT

A rapid, selective and sensitive ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method was developed to simultaneously determine venlafaxine (VEN) and *O*-desmethylvenlafaxine (ODV) in human plasma. Sample pretreatment involved a one-step extraction with diethyl ether of 0.5 mL plasma. The separation was carried out on an ACQUITY UPLC^{TM} BEH C_{18} column with 10 mmol/L ammonium acetate and methanol as the mobile phase at a flow rate of 0.30 mL/min. The detection was performed on a triple–quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. The linear calibration curves for VEN and ODV were both obtained in the concentration range of 0.200–200 ng/mL ($r^2 \geq 0.99$) with the lower limit of quantification (LLOQ) of 0.200 ng/mL. The intra- and inter-day precision (relative standard deviation, R.S.D.) values were less than 13% and the accuracy (relative error, R.E.) was within ±5.3% and ±3.6% for VEN and ODV. The method herein described was superior to previous methods in sensitivity and sample throughput and successfully applied to clinical pharmacokinetic study of venlafaxine sustained-release capsule in healthy male volunteers after oral administration.

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1. Introduction

Venlafaxine, 1-[2-dimethylamino)-1-(4-methoxyphenyl)ethyl] cyclohexanol (VEN) (Fig. 1A), is a non-tricyclic antidepressant, which inhibits the re-uptake of serotonin, noradrenaline and to a lesser extent dopamine [1,2]. Since VEN has less adverse side effects than tricyclic compounds when administrated, it is more suitable for the treatment of depression [2,3]. VEN has several metabolites. In human VEN is metabolized into two minor metabolites and one major active metabolite. About 1% of VEN is desmethylated to *N*-desmethylvenlafaxine (NDV), 16% becomes *N*,*O*-didesmethylvenlafaxine, and 56% is metabolized to *O*-desmethylvenlafaxine (ODV) (Fig. 1B), which has an activity profile on monoamine transporters similar to venlafaxine [4,5]. Therefore, both VEN and ODV levels in plasma are important pharmacokinetic parameters in assessing their efficacy. It is essential to develop a specific, sensitive and rapid method for simultaneous determination of VEN and ODV in human plasma.

A number of analytical methods for the simultaneous determination of VEN and ODV in biological samples have been reported, including HPLC with UV detection [6,7], coulometric detection [8], micellar electrokinetic capillary chromatography (MEKC) [9], and capillary electrophoresis methods [10,11] capable of simultaneously separating enantiomers of VEN and ODV. However these methods tended to lack selectivity. HPLC with fluorescence detection [12,13] had a sensitivity of about 1 ng/mL for both analytes, but these methods needed chromatographic run time longer than 10 min.

Recently, liquid chromatography–mass spectrometry, LC-MS [14] and LC-MS/MS [15–17], were used in simultaneous determination of VEN and ODV. The method Liu et al. [14] developed was advanced in sensitivity, however a total chromatographic run time of 7 min was needed for separation of ODV and NDV having same *m*/*z* in selected ion recording (SIR) mode. Some methods reached sensitivity of 2 ng/mL in plasma and/or a runtime within 3 min [15,16]. Solid phase extraction was employed in one of these methods for cleaning samples [15]. To minimize ion suppression and matrix effect Theron et al. [17] developed an LC-MS/MS method with atmospheric pressure photoionization (APPI).

In order to determine lower plasma concentrations of both VEN and ODV from subjects having lower dose or sustained-release dosage form a more sensitive method is needed. This paper describes a rapid, sensitive and selective UPLC-MS/MS method which enables simultaneous determination of VEN and

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doi:10.1016/j.jchromb.2010.01.007

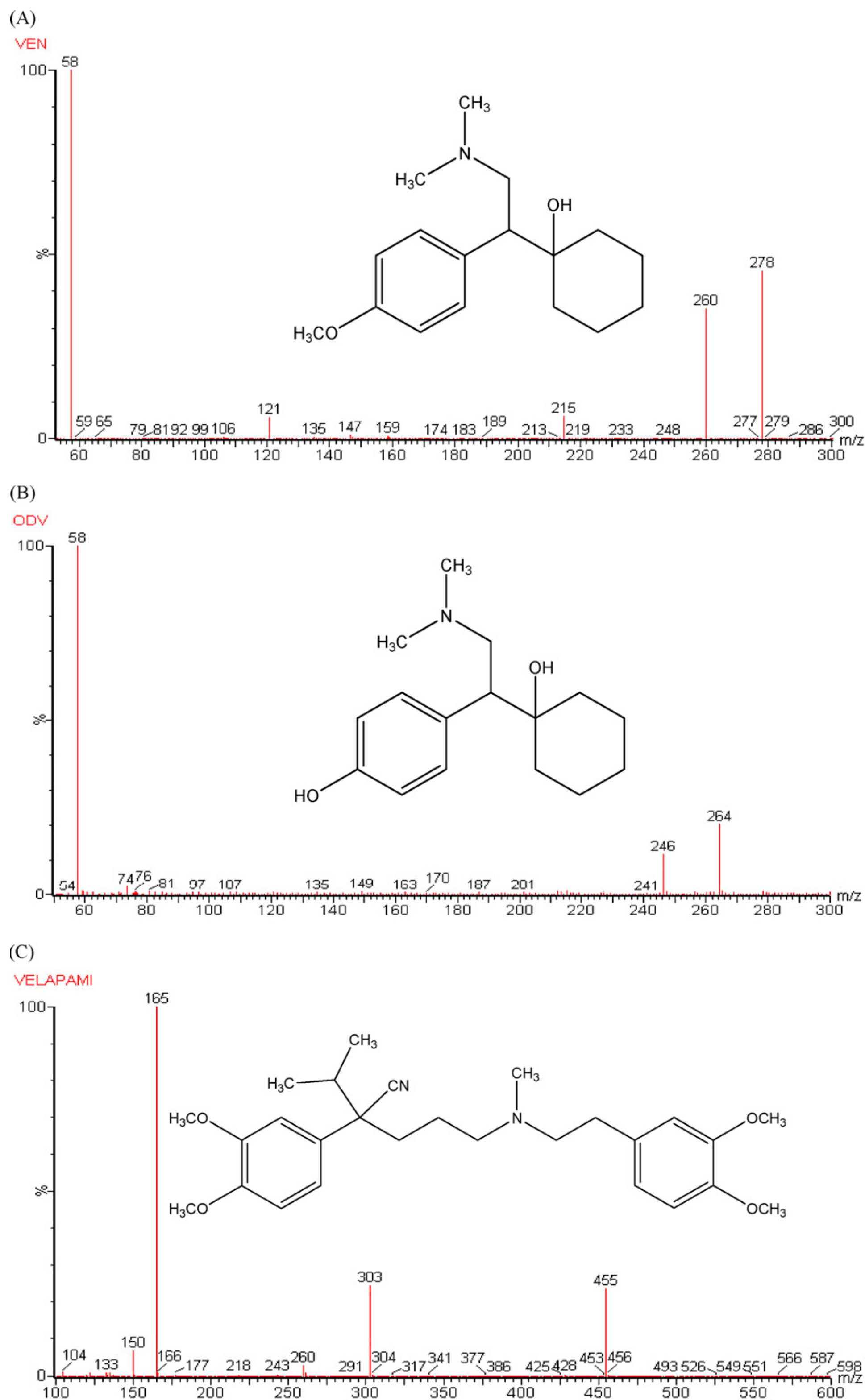

Fig. 1. Full-scan product ion mass spectra of $[M+H]^+$ of venlafaxine (A), *O*-desmethylvenlafaxine (B) and verapamil (C).

ODV with good accuracy at concentrations in human plasma as low as 0.200 ng/mL. The total run time of the method per sample was 2.0 min. This method was fully validated and applied to the pharmacokinetic study in healthy volunteers after oral administration of venlafaxine sustained-release capsule.

2. Experimental

2.1. Chemicals and reagents

The reference standards of venlafaxine hydrochloride (99.8%), *O*-desmethylvenlafaxine (99.8%) and verapamil (internal standard,

I.S., 99.7%) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Methanol of HPLC grade was obtained from Tedia (Fairfield, OH, USA). Ammonium acetate (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The analysis was carried out on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven. An ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed for separation with the column temperature maintained at 40 °C.

The mobile phase was composed of methanol–10 mmol/L ammonium acetate (85:15, v/v). The flow rate was set at 0.30 mL/min. The autosampler temperature was kept at 4 °C and 10 μL of sample solution was injected.

2.2.2. Mass spectrometric conditions

A triple–quadrupole tandem mass spectrometer (Micromass® Quattro micro™ API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of m/z 278 → 58 for VEN, m/z 264 → 58 for ODV and m/z 455 → 165 for I.S., with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 1.2 kV, cone voltage 20 V, source temperature 110 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 400 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 0.258 Pa. The optimized collision energy for VEN, ODV and I.S. was 19, 25 and 27 eV, respectively. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Standard stock solutions of VEN, ODV and I.S. were prepared in methanol at the concentration of 100 μg/mL. And the solutions were then serially diluted with a mixture of methanol–water (50:50, v/v) to provide combined working standard solutions of desired concentrations. I.S. working solution at concentration of 20.0 ng/mL was prepared by diluting the I.S. stock solution with a mixture of methanol–water (50:50, v/v). All the solutions were stored at 4 °C and brought to room temperature before use.

Calibration standards were prepared daily by spiking 25 μL of combined working standard solutions to 500 μL of blank plasma giving concentrations of 0.200, 0.450, 1.00, 2.00, 5.00, 30.0, 100 and 200 ng/mL. The QC samples were prepared with blank plasma at LLOQ, low, middle and high concentrations of 0.200, 0.300, 6.00 and 175 ng/mL and stored at −20 °C after preparation.

2.4. Plasma sample preparation

A 25 μL aliquot of I.S. working solution (20.0 ng/mL) and 200 μL of 1 mol/L sodium hydroxide solution were added to 500 μL of collected plasma sample in 10 mL glass tubes and the mixture was vortexed for 30 s. The sample was extracted with 3 mL of diethyl ether by vortex mixing for 1 min and centrifugation at 3500 × g for 10 min. The upper organic layer was then transferred into another clean glass tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of methanol–water (85:15, v/v), followed by vortexing and centrifugation at 4000 × g for 10 min. The supernatant was transferred into a glass vial, and an aliquot of 10 μL was injected onto the UPLC-MS/MS system.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery and stability according to the FDA guideline for validation of bioanalytical methods [18].

2.5.1. Selectivity

The selectivity was evaluated by comparing the chromatograms of six different batches of normal blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with VEN, ODV, I.S. and plasma sample obtained after oral dose of venlafaxine sustained-release capsule.

2.5.2. Linearity and LLOQ

The calibration curves of VEN and ODV were both constructed using standard plasma samples at eight concentrations in the range of 0.200–200 ng/mL with weighted ($1/x^2$) least squares linear regression. The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy (relative error, R.E.) within ±20% and a precision (relative standard deviation, R.S.D.) below 20% can be obtained.

2.5.3. Precision and accuracy

The intra-day precision and accuracy were evaluated by determining a replicate analysis of QC samples of VEN and ODV on the same day. The run consisted of a calibration curve and six replicates of each LLOQ, low, mid, and high concentration QC samples. For determining the inter-day accuracy and precision, analysis of three batches of QC samples was performed on different days.

2.5.4. Extraction recovery and matrix effect

The extraction efficiency of VEN and ODV was determined by analyzing six replicates of plasma samples at three QC concentration levels of 0.300, 6.00 and 175 ng/mL for each of VEN and ODV. The extraction recovery was calculated by comparing the peak areas obtained from extracted spiked samples with those of unextracted spiked samples at corresponding concentrations. To evaluate the matrix effect, VEN and ODV at three concentration levels were added to the extract of 0.5 mL of blank plasma, dried and reconstituted with 100 μL of methanol–water (85:15, v/v). The corresponding peak areas (A) were compared with those of the standard solutions containing equivalent amounts of the two compounds dried directly and reconstituted with the same solvent (B). The ratio ($A/B \times 100$)% was used to evaluate the matrix effect. The extraction recovery and matrix effect of I.S. were also evaluated using the same procedure.

2.5.5. Stability

The stability of VEN and ODV in human plasma was assessed by analyzing replicates ($n = 5$) of low, mid and high QC samples during the sample storage and processing procedures. QC samples were stored at −20 °C for 30 days and at ambient temperature for 4 h to determine long-term and short-term stability, respectively. The freeze/thaw stability was determined after three freeze/thaw cycles. Post-preparation stability was estimated by analyzing QC samples at 0 and 8 h in the autosampler at 4 °C.

2.6. Pharmacokinetic study

The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their

signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. One venlafaxine sustained-release capsule (containing 75 mg venlafaxine hydrochloride) was administered to each healthy male volunteer after 12 h fasting. Blood samples were collected into sodium heparin-containing tubes before and 1.0, 2.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 24.0, 48.0 and 72.0 h post-dosing [15–16,19]. The plasma was immediately separated by centrifugation and stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. UPLC-MS/MS condition optimization

UPLC-MS/MS operation parameters were carefully optimized for the determination of VEN and ODV. The mass spectrometer was tuned in both positive and negative ionization modes with ESI for both VEN and ODV. The signal intensity obtained in positive ionization mode was much greater than that in negative ionization mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ m/z 278, 264 and 455 for VEN, ODV and I.S., respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecules of the two compounds and I.S. The product ion scan spectra (Fig. 1) showed high abundance fragment ions at m/z 58, 58 and 165 for VEN, ODV and I.S., respectively. The collision gas pressure and collision energy of collision induced decomposition (CID) were optimized for maximum response of the fragmentation of the two analytes. Multiple reaction monitoring (MRM) using the precursor → product ion transition of m/z 278 → m/z 58, m/z 264 → m/z 58 and m/z 455 → m/z 165 was employed for quantification of VEN, ODV and I.S., respectively. No cross-talk was observed between the MRMs of analytes. Compared with LC-MS method (SIR) LC-MS/MS method (MRM) provides higher selectivity. In the VEN case the protonated ion of NDV (another metabolite of VEN) has m/z (264) same as ODV. NDV would interfere in the determination of ODV in SIR mode. Therefore, to avoid the interference a chromatographic separation between the two metabolites before determination was required in an LC-MS method with SIR mode [14]. In the present method with MRM no fragment ion at m/z 58 could be formed from NDV as no dimethylamino group exists in its structure. Therefore, the transition of m/z 264 → m/z 58 was specific for ODV.

The separation and ionization of VEN, ODV and I.S. were affected by the composition of mobile phase. Therefore, the selection of mobile phase is important for improving peak shape, detection sensitivity and shortening run time. Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. Much lower detection response was presented when acetonitrile was adopted. Therefore methanol was chosen as the organic phase. The proportion of methanol in mobile phase affected the peak shape, with 85% methanol generating the best result. When formic acid was added in the mobile phase, the retentions of VEN and ODV on the column were weak and the peak shapes were unacceptable. The ionization of VEN, ODV and verapamil was increased by adding ammonium acetate in the mobile phase. Both analytes and I.S. were found to have highest response and better peak shapes in the mobile phase containing 10 mmol/L ammonium acetate. This binary mobile phase is simpler than those reported in the literature [17], which contained methanol, acetonitrile and buffer. Under the optimal conditions, the total run time for each sample was only 2.0 min, with symmetric peak shape and high sensitivity. This is the shortest analysis time reported so far for simultaneous determination of VEN and ODV, to which both the fast UPLC and the selective MRM contributed. This method meets the requirement for high sample throughput in bioanalysis.

Table 1
Precision and accuracy for the determination of VEN and ODV in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Analyte	Concentrations (ng/mL)		R.S.D. (%)		R.E. (%)
	Nominal	Measured (mean ± S.D.)	Intra-day	Inter-day	
VEN	0.200	0.211 ± 0.019	8.7	9.8	5.3
	0.300	0.293 ± 0.025	4.6	12.3	-2.3
	6.00	5.96 ± 0.32	4.8	8.4	-0.7
	175	171 ± 8.8	5.3	3.2	-2.1
ODV	0.200	0.207 ± 0.020	9.9	4.0	3.6
	0.300	0.302 ± 0.027	7.3	12.8	0.7
	6.00	6.21 ± 0.39	5.3	11.4	3.6
	175	176 ± 8.8	5.2	6.0	0.6

3.2. Method validation

3.2.1. Selectivity

Selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with those of the corresponding spiked plasma batches. As shown in Fig. 2, no interference from endogenous substance or other metabolites was observed at the retention time of VEN, ODV and verapamil.

3.2.2. Linearity and LLOQ

The calibration curves of VEN and ODV (peak area ratios of analytes to I.S. vs. the concentrations of analytes in human plasma) were both linear over the concentration range tested (0.200–200 ng/mL). Typical equations for the calibration curves are: $y = 2.22 \times 10^{-2}x + 7.83 \times 10^{-4}$, $r = 0.996$ for VEN and $y = 6.81 \times 10^{-2}x + 2.51 \times 10^{-4}$, $r = 0.997$ for ODV, respectively. The LLOQ for the two analytes was 0.200 ng/mL in plasma, which was much lower than those reported in literatures [14–17].

3.2.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy for VEN and ODV from QC samples are summarized in Table 1. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of FDA where the R.S.D. determined at each concentration level is required to be not exceeding 15% (20% for LLOQ) and R.E. within ±15% (±20% for LLOQ) of the actual value [18].

3.2.4. Extraction recovery and matrix effect

The extraction recoveries of VEN and ODV from human plasma QC samples were 92.4 ± 5.1%, 86.4 ± 5.3%, 91.6 ± 4.0% and 88.4 ± 6.1%, 89.4 ± 6.3%, 90.6 ± 4.5% at concentration levels of 0.300, 6.00 and 175 ng/mL, respectively, and the mean extraction recovery of verapamil was 94.6 ± 4.5%. Several extraction solvents such as ethyl acetate, cyclohexane, hexane, diethyl ether and diethyl ether-dichloromethane were investigated, and it was found that diethyl ether extracted the analytes more efficiently. Moreover, diethyl ether evaporated to dryness more quickly. In terms of matrix effect, all the ratios defined as in Section 2 were between 85% and 115%. No significant matrix effect for VEN and ODV was observed.

3.2.5. Stability study

The results from all stability tests are presented in Table 2, which demonstrate a good stability of VEN and ODV over all steps of the determination. The method is therefore proved to be applicable for routine analysis.

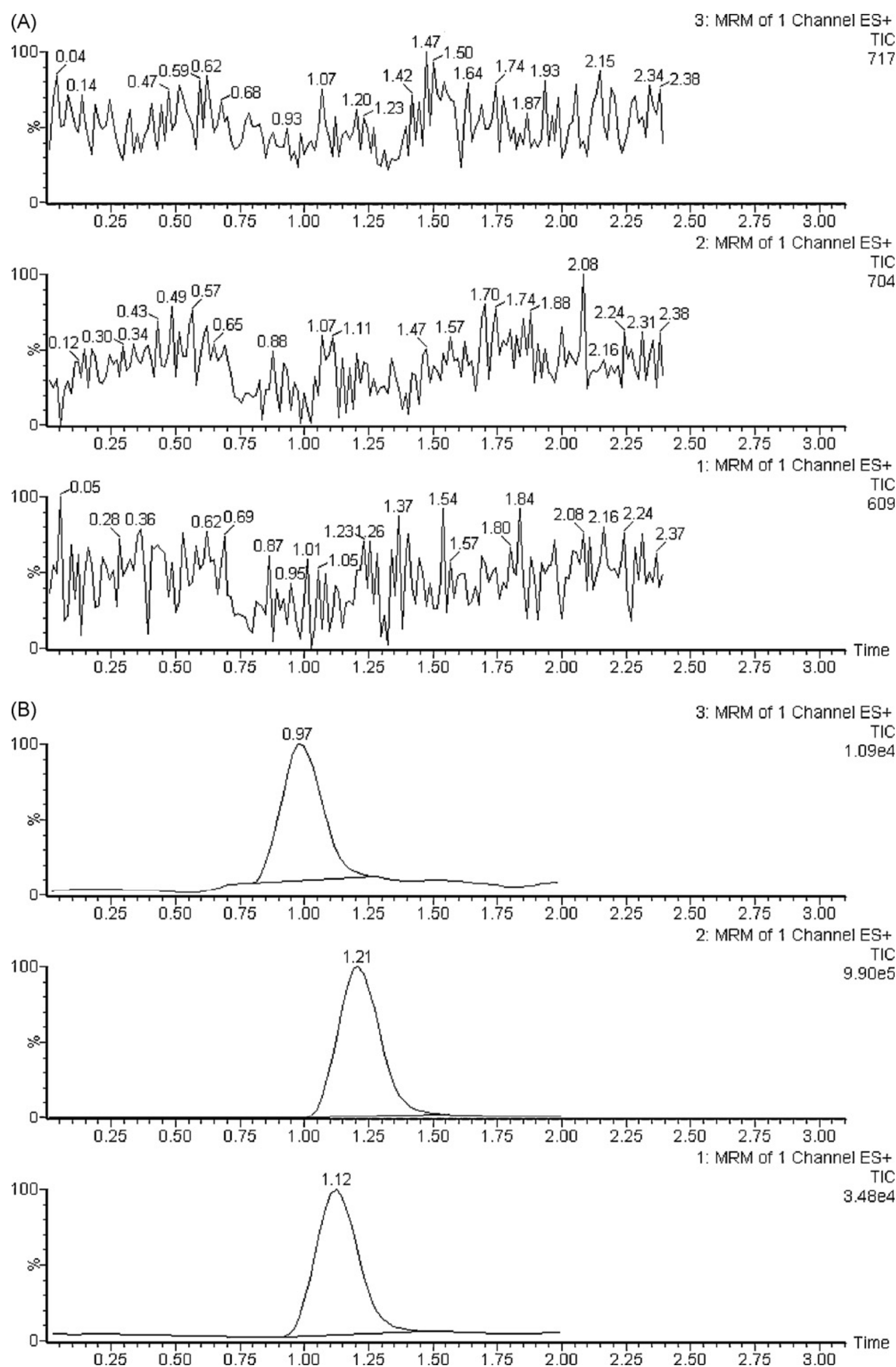

Fig. 2. Representive MRM chromatograms of *O*-desmethylvenlafaxine (channel 3), verapamil (channel 2) and venlafaxine (channel 1) in human plasma samples. (A) Blank plasma sample; (B) blank plasma sample spiked with *O*-desmethylvenlafaxine and venlafaxine at the LLOQ of 0.200 ng/mL and I.S. (20.0 ng/mL); (C) plasma sample from a volunteer 6.0 h after oral administration of venlafaxine. The retention times for *O*-desmethylvenlafaxine, I.S. and venlafaxine were 0.97, 1.21 and 1.12 min, respectively.

3.3. Pharmacokinetic application

This validated UPLC–MS/MS method was successfully applied to a pharmacokinetic study of venlafaxine sustained-release capsule in 18 healthy male volunteers following oral administration of 75 mg venlafaxine hydrochloride. The mean plasma concentration–time curve of VEN and ODV in single dose study is shown in Fig. 3.

The maximum plasma concentration (C_{max}) was 57.8 ± 28.2 ng/mL for VEN and 83.5 ± 32.4 ng/mL for ODV. The time of maximum plasma concentration (T_{max}) was 6.1 ± 1.4 h for VEN and 9.20 ± 1.6 h for ODV. The area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 1251 ± 362 ng h/mL and 1891 ± 389 ng h/mL, the area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 1358 ± 378 ng h/mL and

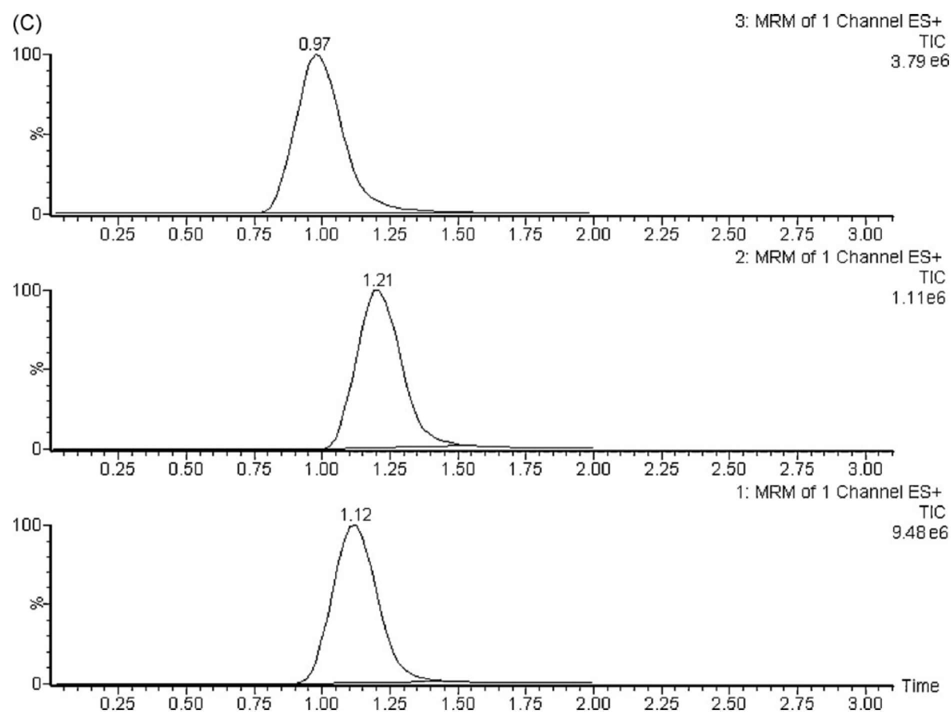

Fig. 2. (Continued)

Table 2
Stability of VEN and ODV in human plasma at three QC levels ($n = 5$).

Stability	Concentrations found (mean ± S.D.)		
	0.300 (ng/mL)	6.00 (ng/mL)	175 (ng/mL)
VEN			
Short-term (room temperature for 4 h)	0.285 ± 0.013	6.07 ± 0.16	174 ± 10
Post-preparative (4 °C for 8 h)	0.310 ± 0.010	5.84 ± 0.12	166 ± 3.9
Three freeze-thaw cycles	0.284 ± 0.017	6.08 ± 0.43	171 ± 9.5
Long-term (−20 °C for 30 days)	0.280 ± 0.011	5.72 ± 0.17	169 ± 6.3
ODV			
Short-term (room temperature for 4 h)	0.285 ± 0.027	5.90 ± 0.47	174 ± 6.3
Post-preparative (4 °C for 8 h)	0.305 ± 0.026	6.48 ± 0.21	173 ± 14
Three freeze-thaw cycles	0.316 ± 0.010	5.70 ± 0.35	181 ± 4.2
Long-term (−20 °C for 30 days)	0.281 ± 0.028	6.26 ± 0.25	166 ± 3.9

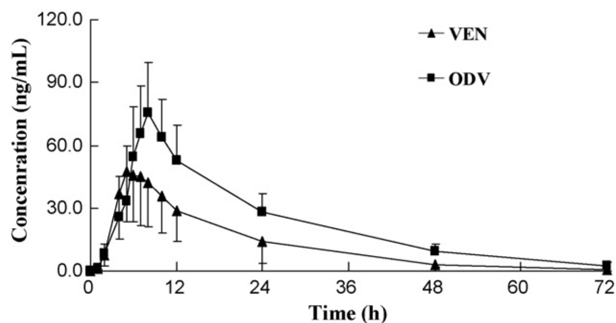

Fig. 3. Mean plasma concentration–time profile of venlafaxine and *O*-desmethylvenlafaxine after oral administration of one venlafaxine sustained-release capsule (containing 75 mg venlafaxine hydrochloride) to 18 healthy male Chinese volunteers (each point represents mean ± S.D.).

1970 ± 411 ng h/mL for VEN and ODV, respectively. The half-life of drug elimination at the terminal phase ($t_{1/2}$) was 9.4 ± 2.6 h for VEN and 13.5 ± 3.3 h for ODV. These pharmacokinetic parameters were consistent with those reported in the literatures [15,16], indicating the applicability of this method to the pharmacokinetic study of VEN.

4. Conclusion

Compared with the analytical methods reported previously, the UPLC-MS/MS method proved to be superior with respect to the sensitivity with an LLOQ of 0.200 ng/mL, satisfactory selectivity and short chromatographic analysis time of 2.0 min. The method has been successfully applied to the pharmacokinetic study of venlafaxine given in sustained-release capsule form to healthy volunteers.

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