

Development of a LC-MS/MS for Quantification of Venlafaxine in Human Plasma and Application to Bioequivalence Study in healthy Korean Subjects

Hyun-Ku Kang¹, Min-A Kang², Hyun-Jin, Kim³, Yoo-Sin Park³, Shin-Hee Kim³ and Ju-Seop Kang^{3*}

¹Department of Psychiatry, Seoul National Hospital, Seoul 110-744, Korea, ²Department of Nursing, College of Nursing, Yonsei University, Seoul 120-752, Korea, ³Department of Pharmacology & Clinical Pharmacology Lab, College of Medicine; Division of Molecular Therapeutics Development, Hanyang Biomedical Research Institute; Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Korea

*Correspondence: J. S. Kang; Tel: +82-2-2220-0652, Fax: +82-2-2292-6686, E-mail: jskang@hanyang.ac.kr

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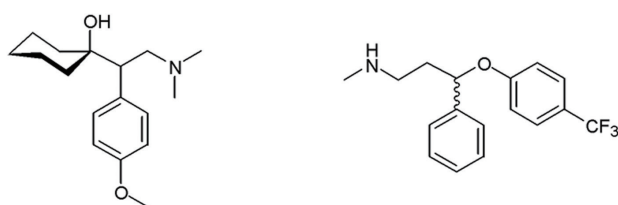
A simple, rapid and selective liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is developed and validated for quantification of venlafaxine in human plasma with simple liquid-liquid extraction step consisted of extraction with ether and dichloromethane for 10 min and mixing with 1 M sodium acetate in human plasma using fluoxetine as an internal standard (IS). The analyte are separated using an isocratic mobile phase consisted of acetonitrile and 5 mM ammonium formate (4/3, v/v) on a isocratic YMC hydrosphere C18 (2.0x50.0 mm, 3.0 μm) column and analyzed by MS/MS in the multiple reaction monitoring (MRM) mode using the transitions of respective [M+H]⁺ ions, m/z 278.2→260.3 and m/z 310.1→148.1 for quantification of venlafaxine and IS, respectively. The standard calibration curves showed good linearity within the range of 1.0-200.0 ng/mL (r²=0.9986, 1/x² weighting). The lower limit of quantification (LLOQ) was 1.0 ng/mL. The retention times of venlafaxine and IS were 0.6 min and 0.7 min that means the potential for the high-throughput potential of the proposed method. In addition, no significant metabolic compounds were found to interfere with the analysis. Acceptable precision and accuracy were obtained for the concentrations over the standard curve range. The validated method was successfully applied to bioequivalence study after 75-mg of venlafaxine sustained-release (SR) capsule in 24 healthy Korean subjects.

Introduction

Venlafaxine (VEN, Figure 1, 1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol hydrochloride) is a phenethylamine bicyclic antidepressant, which show a neuropharmacological profile different from that of existing antidepressant including tricyclic agents.[1] VEN has a mechanism of actions related to selective inhibition of neuronal uptake of norepinephrine, serotonin and, to a lesser extent, dopamine

reuptake without significant affinity for muscarinic, histaminic or α1-adrenergic receptors in the CNS.[2,3] Increased availability of neurotransmitters in the brain caused by their increased release is characteristic of the mechanism of action of nicotine to enhance VEN's antidepressant and precognitive effect,[4] while mecamylamine (nicotinic receptor antagonist) diminished these effects.[5,6] In humans, VEN is metabolized into two minor metabolites, *N*-desmethylvenlafaxine (NDV) and *N,O*-didesmethylvenlafaxine (DDV), and the major active metabolite, *O*-desmethylvenlafaxine (ODV) which presents an activity profile similar to that of VEN.[7] Desvenlafaxine succinate (DVS), the succinate salt of the isolated major active metabolite, is currently under clinical development and may be a new se-

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(A) Venlafaxine

(B) Fluoxetine

Figure 1. Chemical structures of (A) venlafaxine [(RS)-1-[2-dimethylamino-1-(4-methoxyphenyl)-ethyl]-cyclohexanol, MW=277.402 g/mol, C₁₇H₂₇NO₂] and (B) fluoxetine [IS, (RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine, MW=309.33 g/mol, C₁₇H₁₈F₃NO] (from ChemSpider free chemical data base).

rotonin and norepinephrine inhibitor in recent future.[8] The reported method for screening of VEN and its metabolite assay for toxicology and therapeutic drug monitoring in biological fluids include capillary electrophoresis,[9,10] gas chromatography[11] and many conventional HPLC method.[9,12] The UV method is commonly poor sensitivity (200.0 ng/mL) and a long chromatographic run time. Both the methods on HPLC with fluorescence detector are adequately sensitive but the turn-around time for analysis is very long. Recently, a more sensitive LC-tandem mass spectrometry (LC-MS/MS) method was developed to assay VEN and its active metabolite, O-desmethyl venlafaxine (ODV), which could quantify VEN with an LLOQ of below 3.0 ng/mL.[13-15] In this paper, we described a simple, selective and highly sensitive method using HPLC coupled with electrospray ionization (ESI)-MS/MS for the determination of VEN in human plasma and applied to bioequivalence study for 75-mg VEN sustained-release (SR) capsule formulations in 24 Korean volunteers.

Methods

Chemicals and reagents

Venlafaxine [VEN, (RS)-1-[2-dimethylamino-1-(4-methoxyphenyl)-ethyl]-cyclohexanol, MW=277.40 g/mol, C₁₇H₂₇NO₂] and fluoxetine [Figure 1: IS, (RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine, MW=309.33 g/mol, C₁₇H₁₈F₃NO] were obtained from Sigma-Aldrich (St. Louis, MO, USA) and dichloromethane and acetonitrile (HPLC grade) from J.T. Baker (Phillipsburg, NJ, USA). Water was purified using a Milli-Q purification system (Millipore Co., MA, USA). All other chemicals and solvent were HPLC-analytical grades available.

Stock solutions and standards

Primary stock solutions of VEN and IS were prepared with methanol solution to a final concentration of 1.0 mg/mL and 10.0 µg/mL, respectively and both were kept at 4°C until ready to use. A set of six non-zero calibration standards, ranging 1.0-

200.0 ng/mL were prepared in blank human plasma with an appropriate amount of VEN. The quality control (QC) samples were prepared in blank plasma at VEN concentrations of 1.0 (LLOQ), 6.0 (low-middle), 100.0 (high-middle) and 160.0 ng/mL (high). Blank plasma was tested before spiking to ensure that no endogenous interference was found proximal to retention times of VEN and IS.

Preparation for plasma samples

Standard blank plasma was prepared with dilution of stock solution of VEN to its concentration of 1.0-200.0 ng/mL with blank plasma and 500.0 µL aliquot of standard blank plasma was pipetted into a screw cap glass tube. Briefly, 50.0 µL of IS working solution (IS, 10.0 µg/mL) and 100.0 µL of 1 M sodium acetate were added to the 500.0 µL of standard blank plasma and vortexed briefly. A 3.0 mL of mixture of ether and dichloromethane (7:3, v/v) was then added to each sample for liquid extraction, shaken for 10 min. After centrifugation at 4,000 rpm for 5 min, the supernatant stored at deep freezer until ready to use. 1.0 mL of supernatant organic layer was transferred to another set of clean glass tubes and evaporated to dryness with nitrogen gas at 40°C in Speed-Vac system (Savant Co. Holbrook, NY, USA). The residue obtained was dissolved in 500.0 µL of mixture solution of acetonitrile and 0.05% formic acid (1:1, v/v) and vortexed briefly. After brief centrifugation for 5 min at 4,000 rpm, the sample was transferred to autosampler and 3.0 µL of supernatant were directly injected into the LC-MS/MS system.

LC-MS/MS conditions and quantifications

The LC system used was an Agilent 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) chromatograph equipped with an isocratic pump and interfaced with an autosampler (Reliance, Spark Holland, GR, Switzerland). The analytical column was a Hydrosphere C18 (YMC, 2.0x50.0 mm, 3.0 µm) column. The mobile phase consisted of acetonitrile and 5 mM ammonium formate (4:3, v/v) with a flow rate of 250.0 µL/min for analysis. MS analysis was performed using an API 2000 mass spectrometer system (Applied Biosystems, Foster City, CA, USA) equipped with an ESI in positive ionization mode and operated in the multiple-reaction monitoring (MRM) mode for detection of VEN and IS. The ion source parameters were set as the following: curtain gas=39 psi, GS1=50 psi and GS2=50 psi, ion spray voltage=4500 V, ion source temperature=200°C, collision-activated dissociation (CAD)=7.0. This system was set up in MRM mode for monitoring the transitions m/z 278.2 → 260.3 and m/z 310.1 → 148.1 for quantification of VEN and IS, respectively after collision-induced dissociation. Data acquisition and analysis were performed using the analyst software Peak Simple Chromatography Data system version 1.4.1. (Applied Biosystems, Foster City, CA, USA). Total chromatographic run time per sample for analysis is about 1.5 min.

Assay method validation

Assay validation was performed according to the FDA guidance on bioanalytical methods validation.[16,17] Linearity was determined using a linear least-squares regression with $1/x^2$ weighting, which was performed on the peak area ratios of VEN and IS versus VEN concentrations of the six blank plasma standards. The sensitivity of the method was expressed as the lowest limit of quantification (LLOQ) that could be quantitatively determined with acceptable accuracy and precision. The accuracy and precision were assessed by analyzing four concentrations of QC samples with 1.0-200.0 ng/mL from 5 different validation batches and calculated using one-way ANOVA. The selectivity was performed and six randomly selected blank human samples, which were collected under controlled conditions, were carried through the similar extraction procedure and analyzed to determine the extent to which endogenous plasma components could interfere with the analyte or IS at the retention time. To evaluate the inter-day precision and accuracy, validation control samples with drug concentration of 1.0, 6.0, 100.0 and 160.0 ng/mL VEN and IS (10.0 µg/mL) solutions were analyzed together with one independent calibration curve. The accuracy and precision of the inter-day assay were evaluated at the same concentration and calculated for five different days. Inter- and intra-day precision were expressed as relative standard deviation (RSD). The accuracy was expressed as the percent ratio between the experimental and nominal concentrations for each sample. LLOQ was defined as the lowest plasma concentration of each VEN analyzed with an error of 20.0% or lower that corresponds to a signal 5 times greater than the analytical background noise in our experiment.[16,17]

Bioequivalence study in healthy subjects

To evaluate the applicability of this method, a randomized, single-dose, two-period, two-sequence and crossover design was used for bioequivalence study of VEN in 24 healthy Korean subjects. One capsule of 75-mg VEN SR formulations was randomly given to each subject. Participants had not taken other medications (including OTC regimens) for two weeks before or during period including washout period. The study was performed according to the principles of the Declaration of Helsinki (1997) for biomedical research involving human subjects[18] and the guideline for bioequivalence study.[17] Based on this description, they provided voluntarily written consent before participating in the study. In addition, the Institutional Review Board of Hanyang University Medical Center approved the protocol (HYMC-2007-BE013) before start of the study. Twenty-four volunteers aged between 19.0 and 25.0 years (23.0±2.1 years), with heights between 161.0 and 181.0 cm (168.8±7.2 cm) and body weights between 55.0 and 81.0 kg (64.3±10.5 kg), who were non-alcoholics and free from disease state, were assessed as having a healthy status by clinical evaluations, including a physical examination and routine clinical laboratory analysis. During each period, the participants were hospitalized at the

Hanyang University Medical Center at 5PM and had an evening meal before 8PM. After an overnight fast, they received a test or reference drug (single capsule of 75-mg VEN) at 7AM along with 240.0 mL of water. A regular standard lunch and evening meal were provided at 4.0 and 10.0 h after dosing. Liquids that contained xanthine and acidic beverages, including tea, coffee and cola were not allowed. Before and at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 24.0, 36.0, 48.0 and 72.0 h after dosing, vital signs were recorded. Blood samples (9.0 mL) were withdrawn 15 times via an indwelling catheter into heparin-containing tubes from a suitable antecubital vein. The blood samples were centrifuged at 3,000 rpm for 10 min at room temperature and plasma was stored at -70°C until analysis. The total plasma VEN levels were determined and peak plasma concentration (C_{max}) and time to reach to C_{max} (T_{max}) were determined by observed data from each subject's plasma concentration of VEN versus time plots. Other pharmacokinetic parameters were calculated by noncompartmental PK data analysis using PK Solutions software (PK Solutions 2.0, Summit Research Services, Montrose, Co, USA)[19] as follows. The area under the plasma concentration versus time curve ($AUC_{\infty} = \int C_t \cdot dt + C_{last}/\lambda_z$, λ_z = terminal phase slope) was calculated for the total VEN level (C_t) using the linear trapezoidal rule extrapolated from time 0 to infinite time according to a PK analysis program multiline fitting, where C_{last} was the last measurable concentration and the terminal phase slope (λ_z) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration versus time profile. The terminal half-life ($t_{1/2\beta}$) was calculated by $0.693/\lambda_z$. The first moment versus time curve ($AUMC_{\infty}$) was calculated by integration of time (t) of first moment ($C_t \cdot t$) ($AUMC_{\infty} = \int C_t \cdot t \cdot dt + C_t \cdot t/\lambda_z + C_t/\lambda_z^2$). The mean residence time (MRT) of the VEN in the body was calculated by $AUMC/AUC$ and λ_z indicates the elimination rate constant (k_e).

Results

Separation

The molecular structures of VEN and IS are shown in Figure 1. The sample preparation procedures including the simple liquid-liquid extraction of VEN with 1 M sodium acetate and mixture of ether and dichloromethane for 10 min and centrifugation of extracted sample at 4,000 rpm for 5 min at 4°C. The supernatant organic layer (1.0 mL) was evaporated to dryness with gentle nitrogen gas at 40°C and residue obtained was dissolved in 500.0 µL of acetonitrile with 0.05% formic acid. After centrifugation for 5 min at 4,000 rpm, 3.0 µL of supernatant was directly injected into the isocratic LC-MS/MS system. The chromatograms of A) double blank plasma without VEN and IS, B) blank plasma with 10.0 µg/mL of IS, C) blank plasma spiked with 1.0 ng/mL (LLOQ) of calibration standard of VEN and 10.0 µg/mL of IS, and D) subjects plasma taken 10.0 h after a single oral dose of 75-mg VEN SR capsule spiked with 10.0 µg/mL of IS are shown in Figure 2. The retention times of VEN

and IS are about 0.6 min and 0.7 min, respectively, that means the potential for the high-throughput potential of the proposed method. All participants' blank plasma had shown no significant endogenous peaks that might interfere with analyte, using the proposed extraction procedure and chromatographic and mass spectrometric conditions at the retention time of VEN or IS (Fig. 2A). To avoid the interference from exogenous/endogenous compounds co-eluted with the analytes, MS/MS detection was performed. Ionization of analytes was carried out using the ESI technique with positive polarity and MRM mode.

From full-scan mass spectra via the Q1 mass filter, the protonated molecular ions, $[M+H]^+$, at m/z 278.2 for VEN and m/z 310.2 for IS were chosen for the precursor ion (Fig. 3A and 3C). The MS/MS fragmentation was achieved by introducing the $[M+H]^+$ ions into the second quadrupole (Q2) cell with the best collision energy set of 29.0 eV for VEN and 25.0 eV for IS. After collision-induced dissociation, the MS/MS transition m/z 278.2 \rightarrow 260.4 for VEN and m/z 310.2 \rightarrow 148.1 for IS was selected. The most abundant ions in the product ion mass spectrum after collision-induced dissociation at m/z 260.4 for VEN and m/z

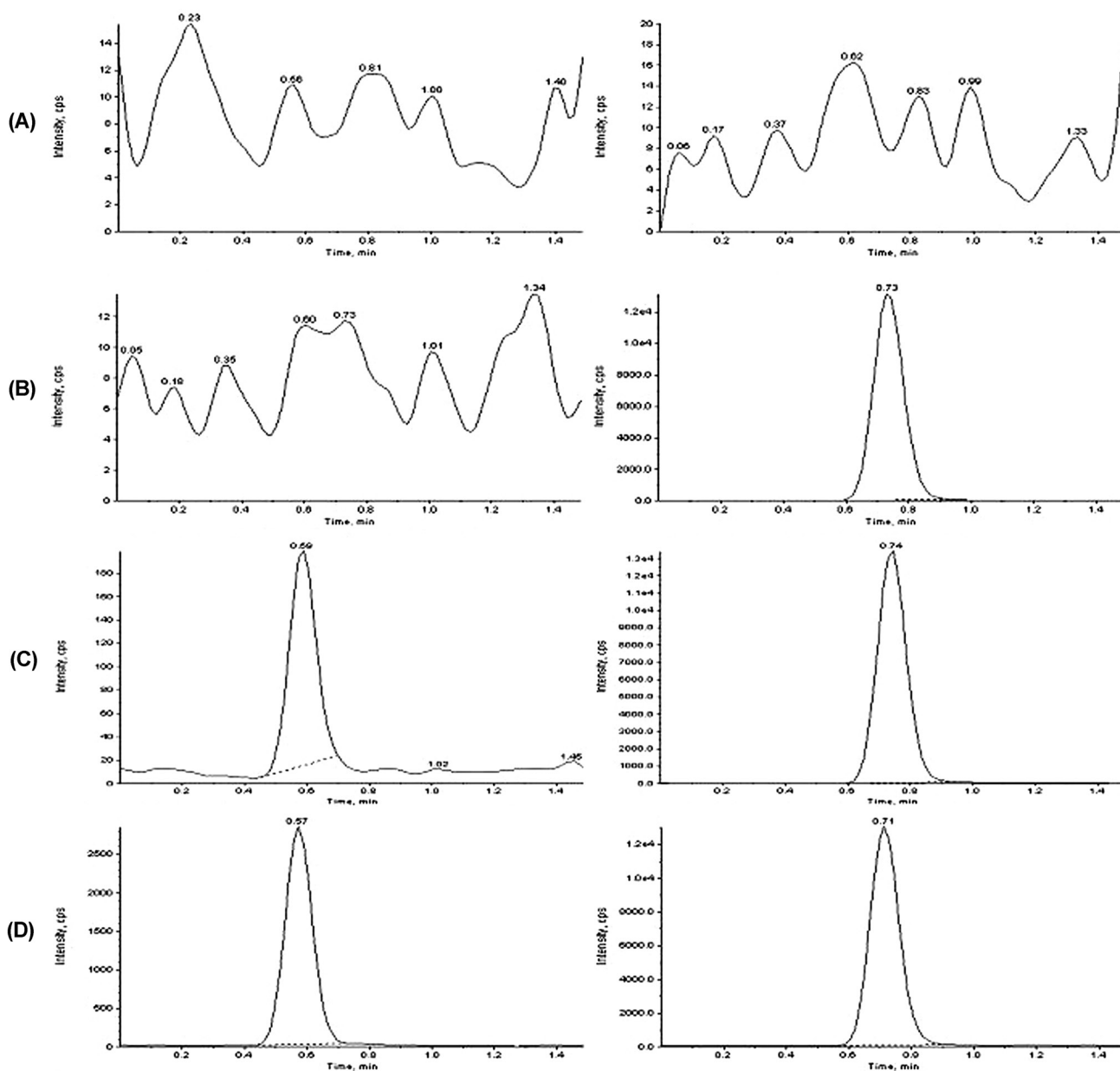


Figure 2. Chromatograms of (A) double blank plasma without venlafaxine and IS, (B) with IS (10.0 $\mu\text{g/mL}$), (C) with venlafaxine (LLOQ, 1.0 ng/mL) and IS (10.0 $\mu\text{g/mL}$), and (D) subject's plasma taken 10.0 h after a single oral dose of 75-mg venlafaxine capsule spiked with IS (10.0 $\mu\text{g/mL}$).

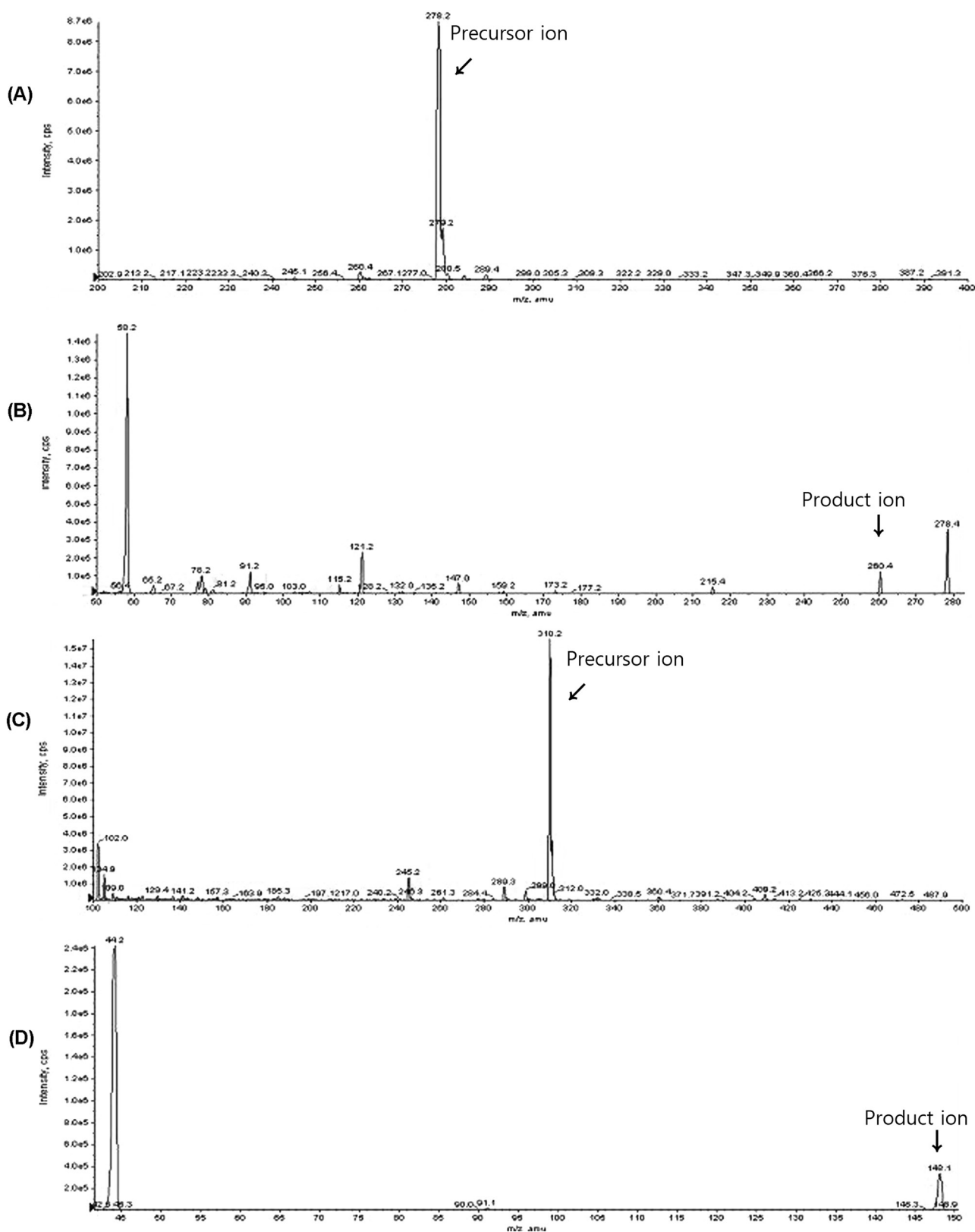


Figure 3. Full-scan mass spectra of precursor (A, C) and product ions (B, D) of venlafaxine (m/z 278.2→260.4) and fluoxetine (m/z 310.2→148.1), respectively.

Table 1. Method validation for the analysis of venlafaxine in human plasma and recovery of venlafaxine and IS (fluoxetine) after the extraction procedure, n=5

Nominal Concentration (ng/mL)	Precision (%RSD) ^b		Accuracy (%)	
	Inter-day	Intra-day	Inter-day	Intra-day
1 (LLOQ ^a)	11.87	5.26	94.30	105.04
6 (low)	5.04	5.03	102.39	101.02
100 (middle)	4.38	2.55	101.94	106.27
160 (high)	5.10	1.80	95.22	97.94

^aLLOQ=lower limit of quantification, ^bRSD=relative standard deviation.

148.1 for IS were monitored for quantification (Fig. 3B and 3D).

Method validation and linearity of calibration

A calibration curve was constructed using a double blank sample (plasma sample without VEN and IS) and six calibration standard samples covering the whole concentration range (1.0-200.0 ng/mL) by the peak area ratio of VEN against IS. The concentrations of VEN were calculated from these area ratios using the calibration curve. The standard calibration curves showed good linearity within the range of 1.0-200.0 ng/mL using least-squares regression analysis ($y=0.0054x+0.0028$, $r^2 \geq 0.9986$, $1/x^2$ weighting). Intra- and inter-day precisions and accuracies were determined by analyzing QC samples with 4 concentrations against a calibration curve, on the same day (n=5) and on different days (n=5). As shown in the Table 1, this method allowed good precision and accuracy. The relative

standard deviation values of both intra- and inter-day were 4.38-11.87% and 1.80-5.26%, respectively. Intra- and inter-day accuracies were 94.30-102.39% and 97.94-106.27%, respectively. Under the described analytical conditions, the LLOQ, defined as the lowest concentration of VEN at which both the precision and accuracy were less than or equal to 20.0%, [16] was 1.0 ng/mL. Because the single oral dose of 75-mg VEN SR capsule resulted in a mean maximal plasma concentration of 48.6 ± 21.3 ng/mL at 6.3 ± 1.5 h for the reference drug and 53.3 ± 27.0 ng/mL at 6.1 ± 2.1 h for test drug, the LLOQ of this method appeared to have enough precision and sensitivity.

Discussion

The proposed method was applied to the determination of VEN concentration in plasma samples for the purpose of establishing bioequivalence study of 75-mg VEN SR capsule formulations in 24 healthy Korean volunteers. The pharmacokinetic parameters for the reference and test drug obtained are described as follows. The profiles of the plasma VEN concentration versus time are shown in Figure 4. Plasma concentrations of VEN were in the standard curve range and remained above the LLOQ (1.0 ng/mL) for the entire sampling period, except for most subjects at 0.5 h and 72.0 h after dosing. Because the bioavailability of orally administered VEN

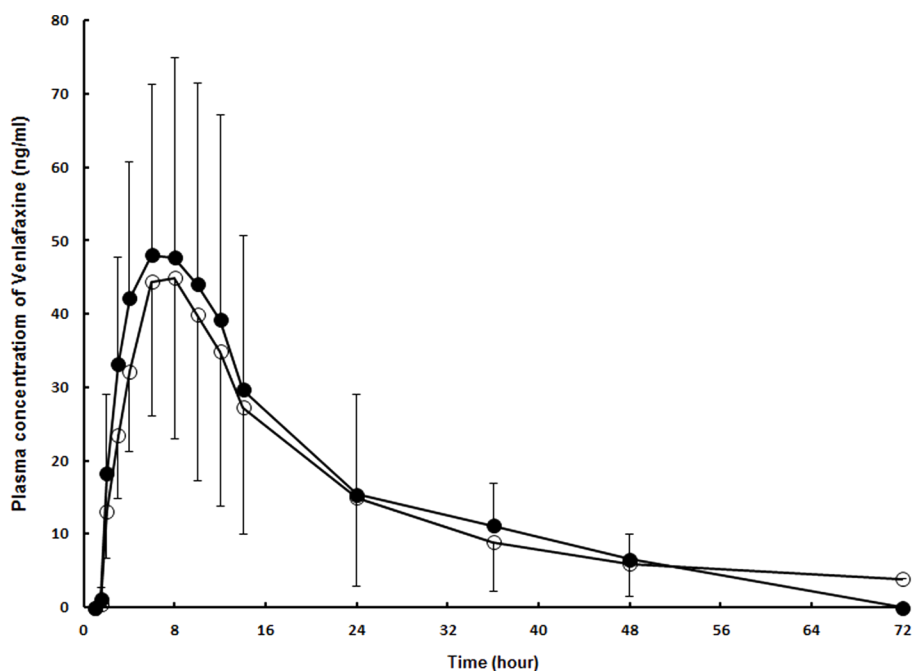


Figure 4. Mean plasma concentrations versus time plots after a single oral dose of 75-mg venlafaxine SR capsule to the 24 healthy male subjects (○: reference, ●: test) (n=24, Mean±SD).

Table 2. Pharmacokinetic parameters (mean±SD, n=24) of two formulations of 75-mg venlafaxine SR capsule based on its blood concentrations in the 24 healthy subjects

Parameters	Reference	Test
AUC _t (ng-h/mL)	797.5±547.5	853.6±610.1
AUC _∞ (ng-h/mL)	864.7±559.4	937.7±644.8
Extrapolation (AUC _{t-∞} /AUC _∞ , %)	9.8±6.4	10.3±5.0
AUMC _t (ng-h ² /mL)	481.774±321.14	526.54±352.01
AUMC _∞ (ng-h ² /mL)	811.77±384.57	954.88±431.68
Vd (L)	33.89±17.19	34.73±19.88
MRT (h)	9.3±5.85	10.0±6.25
C _{max} (ng/mL)	48.6±21.3	53.3±27.0
T _{1/2α} (h)	0.501±0.095	0.61±0.085
T _{max} (h)	6.3±1.5(4.0-10.0)	6.1±2.1(4.0-12.0)
k _a (h ⁻¹)	1.382±0.59	1.135±0.63
T _{1/2β} (h)	9.4±1.9	8.6±2.4
λ _z (k _e , h ⁻¹)	0.0853±0.0018	0.0818±0.0012
CL (L/h)	2.563±0.897	2.381±0.927

AUC_t=area under plasma concentration-time curve from time 0 to t; AUC_∞=area under plasma concentration-time curve from time 0 to infinite time; AUMC_t=area under first moment of plasma concentration-time curve from time 0 to t; AUMC_∞=area under first moment of plasma concentration-time curve from time 0 to infinite time; Vd=apparent volume of distribution; MRT=mean residence time; C_{max}= peak plasma concentration; T_{1/2α}=absorption half-life; T_{max}=time for the C_{max}; k_a=absorption rate constant; T_{1/2β}=elimination half-life; λ_z(k_e)=elimination rate constant; CL=clearance.

is good, mean peak plasma VEN concentrations (C_{max}) were 36.46 ng/mL at 6.0 h for the single oral dose of 75-mg VEN SR capsule in the other study.[20] The plasma profiles of the mean VEN concentration versus time after oral administration of a single dose of both formulations in 24 healthy subjects exhibited some different patterns. The mean estimated pharmacokinetic parameters derived from the plasma concentration profiles of VEN are shown in Table 2. The bioequivalent parameters are similar between test and reference drugs. In previous reports, VEN SR capsules were developed as once-daily alternative to conventional VEN tablets, which require dosing 2 or 3 times. [20,21] Bioavailability of VEN SR capsule formulations after oral administration was good: maximum concentrations are reached with approximately 5.5 h and elimination half-life is approximately 8.0-9.0 h for the ER capsules, compared with 2.0 h for the conventional VEN tablet.[22,23] Our experimental results showed no differences from the results of those studies in normal Korean volunteers. The mean ratio of the AUC_∞ divided by AUC_t was above 9.8 and 10.3% for reference and test drug, respectively. The 90% confidence intervals (CI) of the test/reference percentage ratios were 104.10% (95.56-113.53%) for C_{max} and 107.60% (102.40-113.22%) for AUC_t. Conclusively, the treatments examined would be judged as bioequivalence on the basis of a strict interpretation of the superimposed profiles of drug level in blood after single dose of two kinds of VEN SR capsule formulations.

The proposed method of combining a simple liquid-liquid extraction procedure and a sensitive LC-ESI-MS/MS method provided a rapid and sensitive detection for VEN in human plasma. We achieved shorter retention times (0.6 min for VEN, 0.7 min for IS) and smaller volumes (0.5 mL) of human plasma with sufficient LLOQ (1.0 ng/mL) for application in bioequivalence studies in human subjects. The precision and accuracy for calibration and QC samples were well within the acceptable limits. This method was sensitive enough to analyze plasma VEN concentrations up to 72.0 h after dosing and provided us with a successful application to bioequivalence study of the two kinds of 75-mg VEN SR capsule formulation in 24 healthy Korean volunteers.

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Conflict of interest

The authors have indicated that they have no conflicts of interest with regard to the content of this article.

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