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Short communication

Liquid chromatography/tandem mass spectrometry method for the simultaneous determination of vardenafil and its major metabolite, N-desethylvardenafil, in human plasma: Application to a pharmacokinetic study

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ABSTRACT

A rapid and sensitive LC–MS/MS method for the determination of vardenafil and its major metabolite, N-desethylvardenafil, in human plasma using sildenafil as an internal standard was developed and validated. The analytes were extracted from 0.25-mL aliquots of human plasma by liquid–liquid extraction, using 1 mL of ethyl acetate. Chromatographic separation was carried on a Luna C_{18} column (50 mm × 2.0 mm, 3 μm) at 40 °C, with an isocratic mobile phase consisting of 10 mM ammonium acetate (pH 5.0) and acetonitrile (10:90, v/v), a flow rate of 0.2 mL/min, and a total run time of 2 min. Detection and quantification were performed using a mass spectrometer in the selected reaction-monitoring mode with positive electrospray ionization at m/z 489.1 → 151.2 for vardenafil, m/z 460.9 → 151.2 for N-desethylvardenafil, and m/z 475.3 → 100.1 for the internal standard (IS), respectively. This assay was linear over a concentration range of 0.5–200 ng/mL with a lower limit of quantification of 0.5 ng/mL for both vardenafil and N-desethylvardenafil. The coefficient of variation for the assay precision was <13.6%, and the accuracy was >93.1%. This method was successfully applied to a pharmacokinetic study after oral administration of vardenafil 20 mg tablet in Korean healthy male volunteers.

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1. Introduction

Vardenafil, 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-*f*]-[1,2,4]triazin-4-one (Fig. 1) is a potent and highly selective inhibitor of phophodiesterase type 5 (PDE5) and is used clinically for the improvement of erectile dysfunction [1].

After oral administration, vardenafil is rapidly absorbed. The time (T_{max}) to reach maximum plasma concentration (C_{max}) is usually 0.5–2 h, and the terminal half-life is 4–5 h [2,3]. Vardenafil has a mean absolute bioavailability of ∼15% in humans [4]. It is extensively metabolized, with more than 14 identified metabolites. Metabolism is predominantly by cytochrome P450 (CYP) 3A4 and to a lesser extent by CYP3A5 and CYP2C isoforms. The major circulating metabolite, N-desethylvardenafil, arises from desethylation at the piperazine moiety of vardenafil (Fig. 1). The contribution of N-desethylvardenafil to the overall efficacy of vardenafil is estimated at ∼7% [5]. Recently, it has been reported that the maximum rate (V_{max}) of N-desethylvardenafil formation from vardenafil is higher in human liver microsomes that are heterozygous (n = 9) rather than homozygous (n = 6) for the *CYP3A5*3* allele, compared with the other PDE5 inhibitors, sildenafil and udenafil [6]. Therefore, the simultaneous determination of vardenafil and its active metabolite, N-desethylvardenafil, in biological fluids is necessary for pharmacokinetic and/or pharmacogenomic studies of vardenafil.

A few analytical methods for the determination of vardenafil in tablet formulations have been reported; these methods used micellar electrokinetic capillary chromatography [7] or HPLC with UV detection [8]. LC–MS/MS methods have been employed to determine the PDE5 inhibitors sildenafil, vardenafil, and tadalafil in dietary supplements [9–11]. However, these methods usually cannot be applied directly to the assay of vardenafil in biological samples because of interferences from endogenous materials. Only a few analytical HPLC with fluorescence detection [12] and LC/MS/MS methods [13,14] for determination of vardenafil and/or its active metabolite in biological samples have been reported. Cheng et al. [12] have developed and validated an HPLC method with fluorescence detection to measure vardenafil in rat plasma. Liu et al. [13] have reported an LC–MS/MS method that uses

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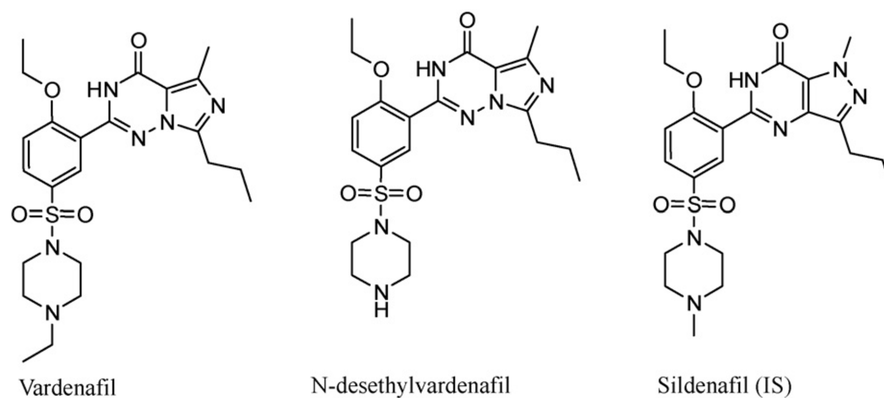

Fig. 1. Chemical structures of vardenafil, N-desethylvardenafil, and sildenafil (IS).

solid phase extraction to determine vardenafil in 1-mL aliquots of human plasma and urine. Zimmer and Muller [14] have compared two methods reverse phase-LC–MS/MS and turbulent flow chromatography–MS/MS to determine vardenafil and its active metabolite in dog plasma.

In this study, we developed a rapid and sensitive LC–MS/MS method for simultaneously determining vardenafil and N-desethylvardenafil in human plasma, using liquid–liquid extraction. This method was successfully applied to a pharmacokinetic study of vardenafil (Levitra®) 20 mg oral tablet in 12 Korean healthy male volunteers.

2. Experimental

2.1. Chemicals and materials

Vardenafil dihydrochloride salt, N-desethylvardenafil, and sildenafil citrate, as an internal standard (IS) for LC–MS/MS analysis, were purchased from Toronto Research Chemicals (North York, ON, Canada). The purities of vardenafil dihydrochloride salt, N-desethylvardenafil, and sildenafil citrate were all >98.5%. Ammonium acetate was from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest analytical grade available. Drug-free human heparinized plasma was obtained from the Clinical Trial Center of Busan Paik Hospital (Busan, South Korea).

2.2. Preparation of standards and quality controls (QCs)

Stock solutions of vardenafil (as a free salt), N-desethylvardenafil, and the IS were prepared by dissolving 1 mg/mL of each, respectively, in methanol. The IS stock solution was further diluted to 400 ng/mL in methanol for routine use. The stock solutions of vardenafil and N-desethylvardenafil were serially diluted with methanol, and these diluted solutions were added with 20 μL of IS solution (400 ng/mL) to drug-free human plasma to produce final concentrations of 0.5, 1, 2, 5, 10, 20, 50, and 200 ng/mL for vardenafil and N-desethylvardenafil, respectively. On the day of analysis, calibration graphs for vardenafil and N-desethylvardenafil in human plasma were derived from the peak area ratio of vardenafil or N-desethylvardenafil to the IS, using linear regression with $1/x$ as a weighting factor. Quality control samples were assayed along with each batch of plasma samples.

The QC samples were prepared in 225 μL of blank human plasma by adding 25 μL of the diluted stock solutions, respectively, to give vardenafil and N-desethylvardenafil concentrations of 0.5, 1, 20, and 200 ng/mL. The QC samples were used to evaluate the intra- and inter-day precision and accuracy of the method. All prepared plasma samples and stock solutions were stored at −80 °C (Revco freezer ULT 1490 D-N-S; Western Mednics, Asheville, NC, USA).

2.3. Sample preparation

A 0.25-mL aliquot of plasma sample was spiked with 20 μL of IS (sildenafil, 400 ng/mL). After vortexing, 1 mL of ethyl acetate was added, and the mixture was vortexed again and centrifuged at $3000 \times g$ for 10 min at 4 °C. The organic layer was separated and evaporated to dryness at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). The residue was reconstituted with 120 μL of acetonitrile, and a 5-μL aliquot was injected directly into the LC–MS/MS system. All prepared samples were kept in an autosampler at 4 °C until injection.

2.4. Characterization of the product ions using tandem mass spectrometry

One micromolar vardenafil, N-desethylvardenafil, and IS solutions were separately infused into the mass spectrometer at a flow rate of 10 μL/min, to characterize the product ions of each compound. The precursor ions $[M+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major peaks observed in the MS/MS scan were used to quantify vardenafil, N-desethylvardenafil, and the IS.

2.5. Analytical system

The plasma vardenafil and N-desethylvardenafil concentrations were quantified using a PE SCIEX API4000 LC–MS/MS system (Applied Biosystems, Foster City, CA, USA), equipped with an electrospray ionization interface used to generate positive ions $[M+H]^+$. The compounds were separated on a reversed-phase column (Luna C_{18}, 50 mm × 2.0 mm i.d., 3 μm particle size; Phenomenex, Torrance, CA, USA) with an isocratic mobile phase consisting of 10 mM ammonium acetate (pH 5.0) and acetonitrile (10:90, v/v). The column and autosampler temperature were maintained at 40 and 4 °C, respectively. The mobile phase was eluted at 0.2 mL/min using an Agilent 1200 series HPLC system (Wilmington, DE, USA).

The optimized ion spray voltage and temperature were set at 5500 V and 500 °C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential, and collision cell exit potential were 106, 60, 10, and 8 V for vardenafil; 71, 60, 10, and 10 V for N-desethylvardenafil; 91, 31, 10, and 10 V for the IS, respectively. Nitrogen gas was used for the nebulizer gas, curtain

gas, and collision-activated dissociation gas, which were set at 100, 60, and 100 psi, respectively. Quantification was performed by selected reaction monitoring of the protonated precursor ion and the related product ion for vardenafil and N-desethylvardenafil, using the IS method with peak area ratios and a linear least-squares regression curve with a weighting factor of $1/x$. The mass transitions used for vardenafil, N-desethylvardenafil, and the IS were m/z 489.1 → 151.2, 460.9 → 151.2, and 475.3 → 100.1, respectively, with a dwell time of 150 ms per transition. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (Version 1.4.1; Applied Biosystems).

2.6. Method validation

The validation parameters were specificity, linearity, sensitivity, accuracy, precision, and matrix effects of the assay and the recovery and stability in human plasma, according to the US Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods [15].

Selectivity was studied by comparing the chromatograms of six different batches of plasma obtained from six subjects, with the plasma samples having been spiked with vardenafil, N-desethylvardenafil, and IS. Calibration curves were prepared by assaying standard plasma samples at vardenafil and N-desethylvardenafil concentrations, ranging from 0.5 to 200 ng/mL.

The linearity of each method matched calibration curve was determined by plotting the peak area ratio (y) of vardenafil or N-desethylvardenafil to the IS versus the nominal concentration (x) of vardenafil or N-desethylvardenafil, respectively. The calibration curves were constructed by weighted ($1/x$) least squares linear regression.

The lower limit of quantification (LLOQ) for vardenafil or N-desethylvardenafil in human plasma was defined as the lowest concentration giving a signal-to-noise ratio of at least 10-fold, acceptable accuracy (80–120%), and sufficient precision (within 20%); this was verified by the analysis of 10 replicates.

Intra- and inter-day accuracy and precision for this method were determined at four different concentration levels on 6 different days, and on each day, six replicates were analyzed with independently prepared calibration curves. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) × 100, and the precision was the relative standard deviation (R.S.D., %).

To evaluate the matrix effect of vardenafil and N-desethylvardenafil on the ionization of the analyte, i.e. the potential ion suppression or enhancement caused by the matrix components, plasma from six different sources was used. If one depicts the vardenafil or N-desethylvardenafil peak areas obtained by direct injection of solvent (or neat) standard solutions as A, the corresponding peak areas for solvent (or neat) standard solutions spiked after extraction into plasma extracts as B, peak areas for solvent (or neat) standard solutions spiked before plasma extraction as C, the matrix effect and extraction recovery values can be calculated as follows [16]:

$$\text{matrix effect } (\%) = \frac{B}{A} 100$$

$$\text{extraction recovery } (\%) = \frac{C}{B} 100$$

The matrix effect and extraction recovery of the IS were evaluated using the same method. All assays were performed in triplicate at concentrations of 1 and 20 ng/mL of vardenafil or N-desethylvardenafil, respectively.

The stability of vardenafil or N-desethylvardenafil in human plasma was assessed by analyzing three replicate samples spiked with 1 and 20 ng/mL, respectively, of vardenafil or N-desethylvardenafil, under four conditions: after short-term storage for 12 h at room temperature; after long-term storage for 60 days at −80 °C; after three freeze–thaw cycles; after sample preparation for 3 h at room temperature. The concentrations obtained were compared with the nominal values of the QC samples. The stabilities of the stock solutions of vardenafil, N-desethylvardenafil, and IS were evaluated after 2 weeks at 4 °C and after 4 months at −80 °C, by comparison with a freshly prepared solution at the same concentration.

2.7. Clinical application

Twelve healthy male volunteers who gave written informed consent took part in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were exclusion criteria. This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, South Korea) and was performed according to the rules of good clinical practice. After an overnight fast, all subjects were given vardenafil 20 mg oral tablet (Byer Korea, Seoul, South Korea). Blood samples (~8 mL) were collected via a cannula before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, and 24 h after administration. The blood samples were immediately centrifuged at 2000 × g for 10 min at 4 °C, and the plasma samples were stored at −80 °C until LC–MS/MS analysis. Pharmacokinetic parameters were calculated by a non-compartmental analysis using WinNonlin Professional software (Version 5.2, Pharsight Corp., Mountain View, CA, USA). The total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated using the trapezoidal rule–extrapolation method. The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the experimental data.

3. Results and discussion

3.1. Mass spectrometry

In the positive ion mode, both vardenafil and N-desethylvardenafil gave protonated molecular ions, $[M+H]^+$, as the major species. The fragmentation patterns of the protonated molecular ions were evaluated by increasing the collision energy. The product ion spectra and fragmentation patterns for vardenafil, N-desethylvardenafil, and the IS are shown in Fig. 2, where the greatest intensity was observed at m/z 151.2 for vardenafil and N-desethylvardenafil, and at m/z 100.1 for the IS, respectively. The mass parameters were optimized by observing the maximal response of the product ions.

3.2. Chromatographic conditions and sample preparation

To optimize chromatographic conditions, many columns, i.e. C_{18}, C_8, phenyl-hexyl, and CN columns and various composition of mobile phases which show good resolution and symmetric peak shapes of analytes as well as suitable retention times were examined. Thus, Luna C_{18} (50 mm × 2.0 mm i.d.; 3 μm particle size) column and a mobile phase consisting of 10 mM ammonium acetate (pH 5.0) and acetonitrile (10:90, v/v) gave good peak shape and response. A liquid–liquid extraction was used in this assay. Several organic solvents including ethylacetate, ether, dichloromethane, acetone, chloroform, and their mixtures were evaluated. Finally, ethylacetate was found to be optimal, which can produce a clean chromatogram for blank plasma samples and the best recovery,

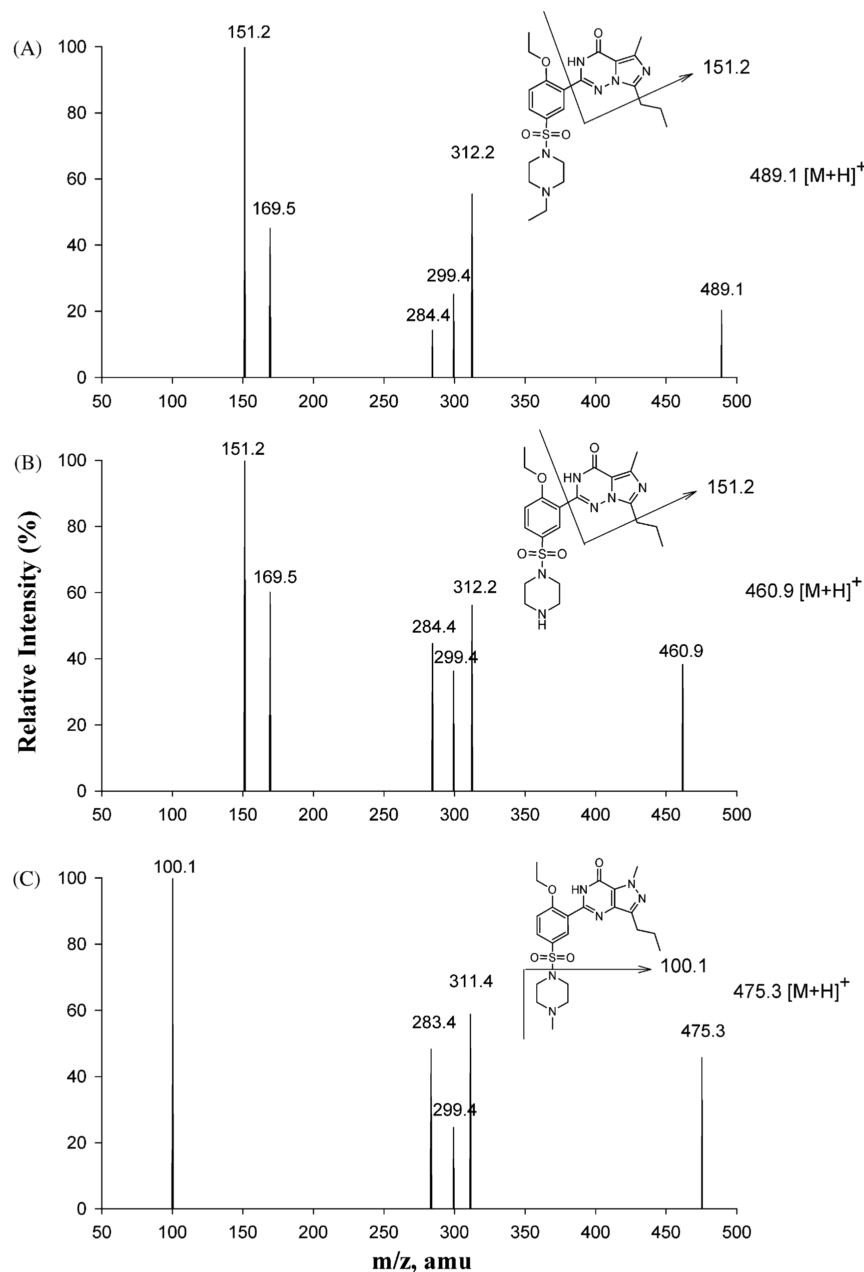

Fig. 2. Product ion mass spectra of vardenafil (A), N-desethylvardenafil (B), and sildenafil (IS) (C).

and the least matrix effect. Although stable isotope labeled internal standards (i.e. vardenafil-d5 or desethylvardenafil-d8) are the first choice, they are not economical and deuterium-labeled compounds may sometimes demonstrate unexpected behavior, such as different retention times or recoveries, than the analyte [17]. Thus, we investigated several compounds to find a suitable IS, and chose a compound being structurally or chemically similar to the vardenafil, sildenafil, as an internal standard in this study.

3.3. Method validation

There were no interfering peaks at the elution times for the analytes (vardenafil, 0.98 min; N-desethylvardenafil, 0.92 min) or the IS (sildenafil, 1.05 min). Fig. 3 shows typical chromatograms for blank plasma; plasma spiked with 5 ng/mL for vardenafil and N-desethylvardenafil, respectively; and plasma collected from a volunteer 0.75 h after the oral administration of a vardenafil 20 mg tablet.

The calibration curves for human plasma provided reliable responses for vardenafil and N-desethylvardenafil from 0.5 to 200 ng/mL. The best linear fit and least-squares residuals for the calibration curve were achieved with $1/x$ weighing factor. The mean correlation coefficient (r) during the validation was 0.9954 (range, 0.9903–0.9989; $n = 6$) for vardenafil and 0.9940 (range, 0.9923–0.9971; $n = 6$) for N-desethylvardenafil in human plasma. The LLOQ for both vardenafil and N-desethylvardenafil was 0.5 ng/mL in human plasma at a signal-to-noise ratio of 10.

The intra- and inter-day precision and accuracy of this assay, which was determined by analyzing six replicates of QC samples at four concentrations on 6 different days, are listed in Table 1. The coefficients of variation for the intra- and inter-day precision were

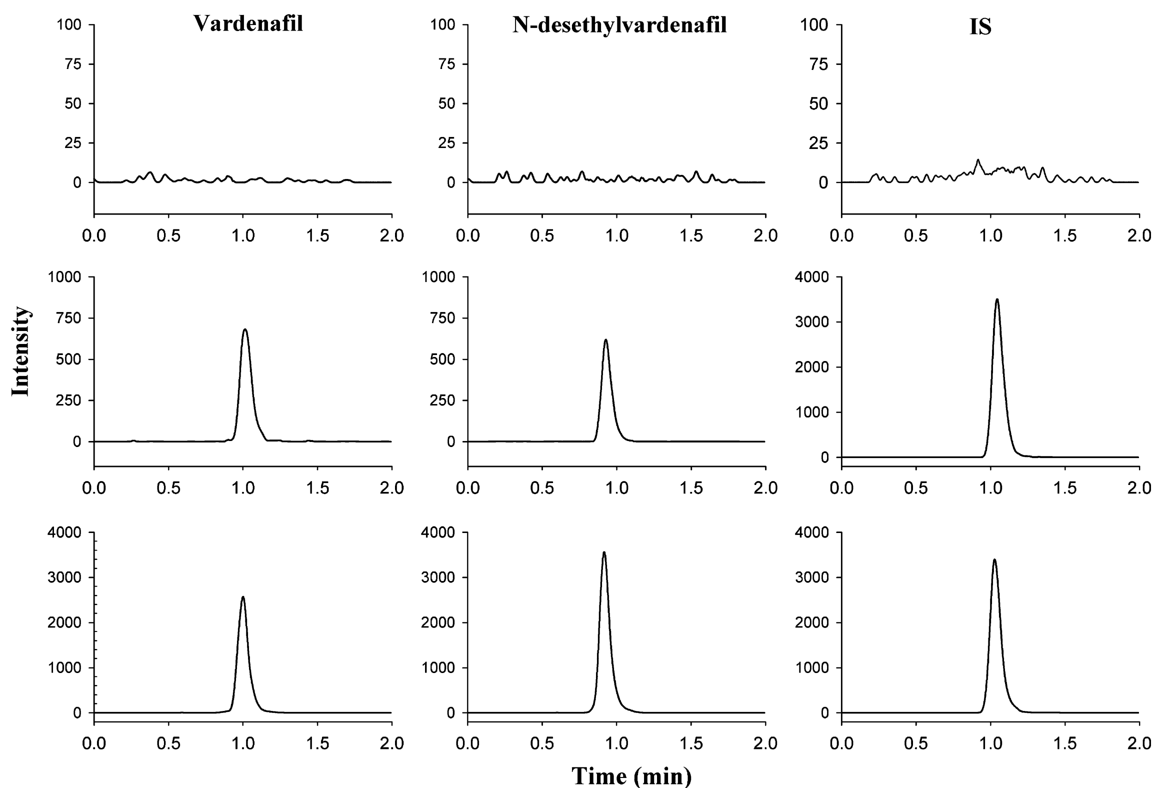

Fig. 3. Representative chromatograms of vardenafil, N-desethylvardenafil, and the IS (sildenafil). (A) Blank plasma. (B) Blank plasma spiked with vardenafil (5 ng/mL), N-desethylvardenafil (5 ng/mL), and the IS. (C) Plasma sample obtained from a volunteer 0.75 h after oral administration of 20 mg of vardenafil (vardenafil: 20.0 ng/mL; N-desethylvardenafil: 23.9 ng/mL).

<13.6% and <12.4%, respectively. The intra- and inter-day accuracies were 93.1–104% and 95.1–110%, respectively.

The percentage of the matrix effect, all the ratios $(A/B \times 100)\%$ defined as in validation procedure section, were between 85 and 115%, which means no significant matrix effect for vardenafil, N-desethylvardenafil, and IS in this method.

The extraction recoveries of vardenafil were 82.6 ± 1.68 and $83.4 \pm 2.22\%$ at 1 and 20 ng/mL, respectively, and the corresponding recoveries of N-desethylvardenafil were 84.8 ± 4.47 and $86.1 \pm 1.43\%$. For the IS, the extraction recovery using an initial concentration of 400 ng/mL was $63.2 \pm 2.00\%$. With a low matrix effect and consistent and reproducible recovery, this assay has proved to be reliable for bioanalysis.

The stock solutions of vardenafil, N-desethylvardenafil, and IS in methanol were stable for 2 weeks at 4 °C and for 4 months at −80 °C; recovered more than 97.8 and 92.4% from samples spiked with the stock solutions. No significant degradation of vardenafil or N-desethylvardenafil in human plasma occurred after short-term

Table 1
Intra- and inter-day precision and accuracy data for assays of vardenafil and N-desethylvardenafil in human plasma ($n = 6$).

Compound	Added (ng/mL)	Precision		Accuracy (%)
		Measured (ng/mL)	R.S.D. (%)	
Intra-day				
Vardenafil	0.5	0.496 ± 0.0316	6.37	99.2
	1	0.995 ± 0.105	10.6	99.5
	20	20.8 ± 1.47	7.08	104
	200	196 ± 16.6	8.47	98.0
N-desethylvardenafil	0.5	0.502 ± 0.0683	13.6	100
	1	1.10 ± 0.0231	2.10	110
	20	18.6 ± 1.16	6.24	93.1
	200	187 ± 12.7	6.80	93.4
Inter-day				
Vardenafil	0.5	0.528 ± 0.0655	12.4	106
	1	1.02 ± 0.0669	6.56	102
	20	19.1 ± 1.48	1.48	95.6
	200	218 ± 10.6	4.84	109
N-desethylvardenafil	0.5	0.550 ± 0.0474	8.61	110
	1	1.03 ± 0.0936	9.12	103
	20	19.0 ± 1.55	8.13	95.1
	200	208 ± 16.4	9.34	104

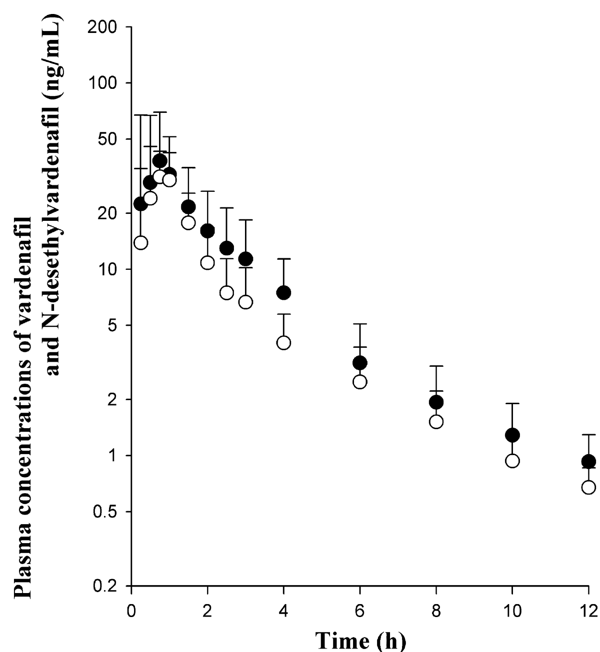

Fig. 4. Plasma concentration–time profiles for vardenafil (●) and N-desethylvardenafil (○) after oral administration of a vardenafil 20 mg tablet to 12 Korean healthy male volunteers. Vertical bars represent standard deviation.

Table 2
Pharmacokinetic parameters of vardenafil and N-desethylvardenafil after single oral administration of vardenafil 20 mg tablet to 12 Korean healthy male volunteers.

Parameters[a]	Vardenafil	N-desethylvardenafil
$AUC_{0-\infty}$ (ng h/mL)	89.2 ± 55.4	65.4 ± 27.2
AUC_{0-t} (ng h/mL)[b]	88.5 ± 58.7	68.2 ± 27.4
Terminal half-life (h)	2.39 ± 0.767	2.87 ± 0.806
C_{max} (ng/mL)	44.1 ± 35.9	38.3 ± 16.5
T_{max}[c] (h)	0.75 (0.25–1)	0.75 (0.5–1)

[a] Values are mean ± standard deviation.
[b] The area under the plasma concentration–time curve from time zero to last sampling time.
[c] Median (ranges).

storage for 12 h at room temperature, long-term storage for 45 days at −80 °C, three freeze–thaw cycles, or sample preparation for 3 h at room temperature, with ±15% deviation between the predicted and nominal concentrations at 1 and 20 ng/mL (data are not shown).

3.4. Clinical application

This LC–MS/MS method was successfully applied to a pharmacokinetic study of vardenafil tablets. The mean plasma concentration–time profiles for vardenafil and N-desethylvardenafil in 12 Korean healthy male volunteers after oral administration of a vardenafil 20 mg tablet are shown in Fig. 4 and some relevant pharmacokinetic parameters are summarized in Table 2. The sensitivity and specificity of this method were sufficient for characterizing the pharmacokinetics of vardenafil and N-desethylvardenafil. The QC samples ranged within 15% of the nominal concentrations, meeting the acceptance criteria of the US FDA for the validation of bioanalytical methods [15]. The mean C_{max} of vardenafil was 44.1 ± 35.9 ng/mL occurring at (T_{max}) 0.75 h (ranges, 0.25–1 h). The terminal half-life and $AUC_{0-\infty}$ values of vardenafil were 2.39 ± 0.767 h and 89.2 ± 55.4 ng h/mL, respectively. The mean C_{max} of N-desethylvardenafil was 38.3 ± 16.5 ng/mL occurring at (T_{max}) 0.75 h (ranges, 0.5–1 h), and the $AUC_{0-\infty}$ value was 65.4 ± 27.2 ng h/mL. The pharmacokinetic parameters of vardenafil and N-desethylvardenafil were similar to those reported in the literature [2,3,5,18].

4. Conclusion

We report the development and validation of a rapid and sensitive LC–MS/MS method with liquid–liquid extraction for the simultaneous determination of vardenafil and N-desethylvardenafil in human plasma, over a concentration range of 0.5–200 ng/mL. This method requires only 0.25 mL of a biological sample, and owing to the simple sample preparation and short run time (2 min), it allows high sample throughput. The method was successfully applied to a pharmacokinetic study of vardenafil and its major metabolite, N-desethylvardenafil.

Acknowledgments

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