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Article

Quantification of Nimesulide in Human Plasma by High-Performance Liquid Chromatography with Ultraviolet Detector (HPLC-UV): Application to Pharmacokinetic Studies in 28 Healthy Korean Subjects

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Nimesulide is a selective COX-2 inhibitor that is as effective as the classical non-acidic nonsteroidal anti-inflammatory drugs in the relief of various pain and inflammatory conditions, but is better tolerated with lower incidences of adverse effects than other drugs. After oral dose of 100 mg nimesulide to western subjects, a mean maximal concentration (C_{max}) of 2.86 ~ 6.5 μg/mL was reached at 1.22 ~ 2.75 h and mean $t_{1/2\beta}$ of 1.8 ~ 4.74 h. This study developed a robust method for quantification of nimesulide for the pharmacokinetics and suitability of its dosage in Korea and compared its suitability with other racial populations. Nimesulide and internal standard were extracted from acidified samples with methyl *tert*-butyl ether and analyzed by high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The 28 healthy volunteers took 2 tablets of 100 mg nimesulide and blood concentrations were analyzed during the 24 h post dose. Several pharmacokinetic parameters were represented: $AUC_{0-infinity}$ = 113.0 mg-h/mL, C_{max} = 12.06 mg/mL, time for maximal concentrations (T_{max}) = 3.19 h and $t_{1/2\beta}$ = 4.51 h. These were different from those of western populations as follows: AUC was 14.5% and C_{max} was 28% that of of Korean subjects and T_{max} and $t_{1/2\beta}$ were also different. The validated HPLC-UV method was successfully applied for the pharmacokinetic studies of nimesulide in Korean subjects. Because the pharmacokinetics of nimesulide were different from western populations, its dosage regimen needs to be adjusted for Koreans.

Introduction

Nimesulide (*N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide) is a relatively COX-2 selective, non-steroidal anti-inflammatory drug (NSAID) that is as effective as the classical NSAIDs in the relief of a wide variety of pain and inflammatory conditions, but is better tolerated with lower incidences of adverse effects than other NSAIDs (1–3). Nimesulide shows its pharmacological activity through various mechanisms of action and the major mechanisms concern the selective inhibition of cyclooxygenase-2 (COX-2) with 5 ~ 16-fold selectivity for COX-2 (2, 4–9). COX-1, which regulates gastric cytoprotection and vascular hemostasis, is expressed in many tissues . Inhibition of COX-1 reduces the synthesis of cytoprotective compounds, such as prostacyclin, and may result in unwanted gastrointestinal and renal adverse effects. However, selective inhibition of COX-2 reduces the production of pro-inflammatory prostaglandins, and may provide beneficial effects in inflammation and pain relief, with modest gastrointestinal (GI) toxicity (1, 2). *Ex vivo* measurements in human whole blood after oral administration of 100 mg nimesulide show complete suppression of COX-2 activity and partial reduction in COX-1 activity (10). *In vitro*, nimesulide does not affect prostaglandin synthesis in the bronchial tree (11), where constitutive COX-1 exerts a bronchoprotective role, or in the gastric mucosa (12, 13), where COX-1 preserves mucosal integrity. In contrast, nimesulide markedly affects prostaglandin production in inflammatory exudates (12), where prostaglandin production is mediated by COX-2. Nimesulide has been reported to inhibit histamine release from human basophils and tissue mast cells (14), platelet-activating factor synthesis in phagocyte-stimulated human neutrophils (15) and metalloproteinase synthesis like collagenase and stromelysin (16).

Nimesulide is usually administered orally and the usual dosage is 100 mg twice daily, increasing to 200 mg twice daily, depending on the severity of symptoms and patient response. The pharmacokinetic studies have been performed using various formulations. Some papers have described the pharmacokinetic profile of nimesulide in healthy volunteers after single and multiple administrations (17) and the effects of age and disease on the pharmacokinetic variable (18). Nimesulide is rapidly absorbed from GI tracts. After oral administration of a 100 mg dose to healthy fasting individuals, a mean maximal concentration (C_{max}) of 2.86 ~ 6.50 mg/L was achieved within 1.22 ~ 2.75 h (19). The plasma concentration of nimesulide is usually determined using various high-performance liquid chromatography (HPLC) methods developed by Castoldi *et al.* (20) and Pandya *et al.* (21). Therefore, the aims of present study were to develop and validate a sensitive, robust and simple isocratic HPLC-UV method for quantification of nimesulide in human plasma and to dramatically increase sample throughput and efficiency in analyzing large amounts of plasma samples obtained from clinical pharmacokinetic or bioequivalence studies. We tried to apply this method to study pharmacokinetic studies after single oral doses of 2 tablets of 100 mg nimesulide to 28 healthy Korean volunteers.

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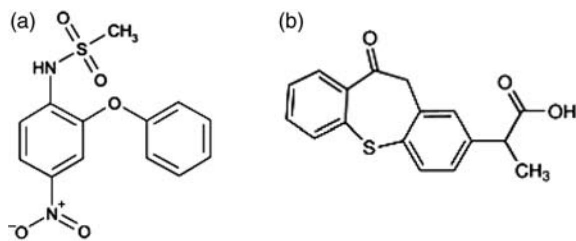

Figure 1. Chemical structures of nimesulide (a; *N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide, MW = 308.311, $C_{13}H_{12}N_2O_5S$) and IS (b; zaltoprofen, 10, 11-Dihydro-a-methyl-10-oxodibenzo[b,f]thiepin-2-acetic Acid, MW = 298.36, $C_{17}H_{14}O_3S$).

Experimental

Chemicals and reagents

Nimesulide ($C_{13}H_{12}N_2O_5S$, MW 308.311 g/mol) and zaltoprofen [internal standard (IS), $C_{17}H_{14}O_3S$, MW 298.356 g/mol] were purchased from Sigma-Aldrich (St. Louis, MO) (Figure 1). Purity was found to be more than 99% in all compounds. Hydrochloric acid, methyl *tert*-butyl ether, methanol and sodium phosphate were all HPLC-grade and purchased from Sigma (St. Louis, MO). All aqueous solutions, including the buffer for the HPLC mobile phase, were prepared with water that was purified by a Milli-Q water purification system (Millipore, Milford, MA). Reference drugs (Zanitip, LG Life Science, South Korea) containing 100 mg nimesulide per tablet were used in this study.

Stock solutions and standards

Primary stock solutions of nimesulide and IS were prepared with methanol solution to final concentrations of 1 mg/mL and 300 μg/mL, respectively, and stored at −20°C. A set of six non-zero calibration standards ranging from 0.2 to 40 μg/mL was prepared by spiking the blank drug-free human plasma containing ethylenediamine tetraacetic acid with an appropriate amount of nimesulide. The quality control samples at four concentration levels (0.2, 0.6, 20 and 36 μg/mL) were prepared in a similar manner to the calibration standards. Blank human plasma was tested before spiking to ensure that no endogenous interference was found at retention times of nimesulide and IS.

Preparation for plasma samples

After dilution of stock solution of nimesulide to its concentration of 0.2 ~ 40 μg/mL with blank plasma, a 0.5 mL aliquot of human plasma was pipetted into a screw cap glass tube. Briefly, 0.1 mL of IS working solution (IS, 300 μg/mL) and 50 μL of 2.0M hydrochloric acid was added to the plasma samples (1 mL), and the nimesulide and IS were extracted with 5 mL of methyl *tert*-butyl ether by shaking in a vortex mixer for 10 min. After centrifugation at 4,000 rpm for 5 min, the upper organic phases were collected and evaporated to dryness, residues were dissolved in 500 μL of a mixture of methanol and 50 mM sodium phosphate (57:43, *v/v*) and 40 μL of aliquots of final extracts were injected into the HPLC column in the HPLC-UV system.

HPLC system and quantifications

The HPLC system was a Waters (, Milford, MA) LC system equipped with a Waters 510 pump, Waters 717 Plus autosampler, Waters TCM column oven and Waters 486 UV detector. The data were acquired and processed with Empower 3 software. The analytical column was a Capsell Pak C18 UG120 (150 × 4.6 mm, 5 μm; Shiseido, Japan). The mobile phase consisted of methanol and 50 mM sodium phosphate (57:43, *v/v*, pH = 6.03); the flow rate was 1.0 mL/min at 35°C and the injection volume was 40 μL. An elution was monitored by the UV detector set at 334 nm and total run time was set to 15 min.

Assay validation

A calibration curve was constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed spiked with IS) and six non-zero samples covering the total range from 0.2 to 40 mg/mL, including the lower limit of quantification (LLOQ). The acceptance criterion for each concentration was 15 % deviation from the nominal value, except for the LLOQ, which was set at 20%. Plots of plasma concentrations versus peak area ratios of nimesulide to IS for calibration range for nimesulide in human plasma were constructed and linear regression lines (weighting factor 1/y2) were used for the determination of nimesulide concentration in plasma samples. The specificity was performed and six randomly selected blank human samples, which were collected under controlled conditions, were carried through a 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 concentrations of 0.2, 0.6, 20 and 40 mg/mL and IS (300 mg/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-day 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. The LLOQ was defined as the lowest plasma concentration of each nimesulide analyzed with an error of 20% or lower that corresponds to a signal 5 times greater than the analytical background noise in our experiment (22, 23). Recovery of nimesulide in plasma was evaluated by comparing the mean detector response of different quality control samples extracted with those prepared by adding the compound to post-extracted drug free plasma at the corresponding concentrations. Similarly, the recovery of IS from plasma was also evaluated.

Pharmacokinetic studies in healthy Korean volunteers

The pharmacokinetic study was conducted according to the revised Declaration of Helsinki for biomedical research involving human subjects (24) and the rules of Good Clinical Practice (25). The protocol of this study was approved by the Institutional Review Board of Hanyang University Medical Center. Twenty-eight healthy male volunteers, 19 ~ 29 years (mean ± SD, 23 ± 2.3 years), with height of 166.1 ~

183.0 cm (mean ± SD, 174.9 ± 5.3 cm), weight of 58.0 ~ 91.1 kg (mean ± SD, 72.5 ± 13.1 kg) and within 15 % of their ideal weight, were recruited by Internet advertisement and participated in this study after giving informed consent. Subjects with a history of drug allergies, renal or hepatic dysfunction, a history of any illnesses of cardiovascular system or alcohol and drug abuse were excluded. Subjects were selected after passing a clinical screening procedure, including physical examination and clinical laboratory tests. All subjects avoided using other drugs for at least 2 weeks before the study and after its completion. They also abstained from alcoholic beverages and xanthine-containing foods and beverages for 48 h prior to each dosing and until the collection of the last blood sample.

The volunteers were hospitalized in Hanyang University Medical Center at 6:00 PM and had an evening meal before 8:00 PM. Subjects received an oral dose of two tablets of 100 mg nimesulide at 7:00 AM along with 240 mL of water after an overnight fasting. Subjects were then placed in a seated position for at least 1 h and fasted for the first 4 h after dose. A standard lunch and evening meal were provided at 4 and 10 h after dose. The subjects were continuously monitored by medical staff throughout the confinement period of the study. Liquid consumption was allowed after lunch, except xanthine-containing or acidic beverages such as tea, coffee and coke. Blood samples (approximately 10 mL) were drawn by indwelling venous catheter into heparin-containing tubes from a suitable antecubital vein before dose and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after dose. The blood samples were centrifuged at 2,000 × *g* for 10 min at 4°C and the plasma was transferred to separate E-tubes to be kept frozen at −70°C until analysis.

The C_{max} and the time for maximal concentrations (T_{max}) were directly determined by visual inspection of the plots from each subject's plasma concentration of nimesulide versus time . Other pharmacokinetic parameters were analyzed and calculated by noncompartmental pharmacokinetic data analysis using PKCAL (a basic interactive computer program for statistical and pharmacokinetic analysis of data) computer software as follows. The area under the plasma concentration versus time curve (AUC_{0-24h}) was calculated by linear trapezoidal method for the time period of 0 to 24 h. The AUC extrapolated to infinity ($AUC_{0-infinity}$) was calculated as follows: $AUC_{0-infinity} = AUC_{0-last} + C_{last}\ /\ \lambda$, λ = slope of elimination phase) was calculated for the total nimesulide level (C_t) using the linear trapezoidal rule extrapolated to infinity, according to a noncomparmental pharmacokinetic analysis where C_t was the last measurable concentration and the last slope (λ) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration versus time profile (26, 27). The first moment versus time curve ($AUMC_{0-infinity}$) was calculated by intergration of time (t) of first moment ($C_t \times t$) ($AUMC = \int C_t \times t \times dt + Cp \times t\ /\ \lambda z + Cp\ /\ \lambda_z^2$). The mean residence time (MRT) of the nimesulide in the body was calculated by AUMC/AUC and λ_z indicates the elimination rate constant (*Ke*) (27–29).

Results and Discussion

The molecular structures of nimesulide and zaltoprofen (IS) are shown in Figure 1. Thus far, published analytical methods of nimesulide determination in plasma apply separation on reversed phases using acidic mobile phases (pH 3 ~ 5.5) and spectrophotometric detection at 230 ~ 290 nm. Those methods demonstrate several disadvantages in the application of the pharmacokinetic studies with bioequivalence testing, such as a complex extraction procedure involving extensive sample preparation with large amounts of solvents, or an IS not available commercially like NS-398 or DRF-4367 (30, 31). However, this method used a different IS (zaltoprofen), different UV-visible spectrum corresponding to 334 nm and a less acidic mobile phase (pH 6.03) than reported methods (30, 32).

Sample handling involved extraction of nimesulide and IS from acidified plasma using methyl *tert*-butyl ether. After solvent evaporation, extract residue was dissolved in the mobile phase and analyzed on a C18-reversed-phase HPLC column under isocratic elution conditions and UV (334 nm) detection. The HPLC system was operated isocratically at a controlled column temperature of 35°C using methanol−50 mM sodium phosphate buffer (57:43, *v*/*v*, pH 6.03) as mobile phase, filtered through a 0.45 μm membrane filter and run at a flow rate of 1.0 mL/min. An injection volume of 40 mL was used for the standard and samples and all determinations were carried out with three to five replicates.

Figure 2 shows typical chromatograms of extracted samples of human plasma without nimesulide or IS (A), plasma spiked with IS (300 μg/mL) (B), plasma spiked with nimesulide (0.2 μg/mL) and IS (300 μg/mL) (C) and plasma drawn from subject on 2 h after oral dose of 2 tablets of 100 mg nimesulide (D). No significant interfering peaks from endogenous materials in the plasma were found at the retention time. A sharp symmetrical peak corresponding to nimesulide and IS was well separated and clear on the chromatogram with stable retention time.

The developed and validated method proved to be efficient for the determination of nimesulide in human plasma and can readily be applied to pharmacokinetic or bioavailability studies. The proposed method was simple, rapid, sensitive, specific and reproducible. Nimesulide and IS were separated well from other plasma components with retention times of 6.8 and 9.9 min, respectively. The linearity of nimesulide curves ranged from 0.2 to 40 μg/mL (y = 0.03776× − 0.00167, *r*2 = 0.9994, 1/x weighting). The LLOQ for nimesulide was 0.1 μg/mL in the plasma, the accuracy was 108.5% and the intra-day and inter-day precision were 1.04 and 5.57 %, respectively. The intra-day accuracy ranged from 97.25 to 106.71%, while inter-day accuracy ranged from 97.96 to 108.50%, and the recovery of nimesulide and IS were determined and the averages were excellent. The AUC measured from 0 to the last sampling time (24 h) was approximately 94.5% of the value of AUC extrapolated from 0 to infinity, which means the analytical method is suitable for pharmacokinetic studies (Table I). The mean plasma concentration-time curves of nimesulide obtained from the blood of 28 normal subjects are shown in Figure 3. Several calculated pharmacokinetic parameter values are shown in Table II and were inconsistent with previously reported values (17, 19, 33,34). The pharmacokinetic assessment of nimesulide in this study was differerent from that reported by Bernareggi (19), who showed, after oral dose of 100 mg tablet, values of C_{max} at 2.78 ~ 7.48 μg/mL, $AUC_{0-infinity}$ at 21.99 ~ 70.07 μg-h/mL and T_{max} at 2.0 ~ 3.5 h. Gandini *et al.* (1991) obtained mean values of C_{max} at 6.17 μg/mL, $AUC_{0-infinity}$ at 50.93 μg-h/mL and T_{max} at 2.5 h after oral dose of 100 mg tablet (33). In the current study, after oral dose

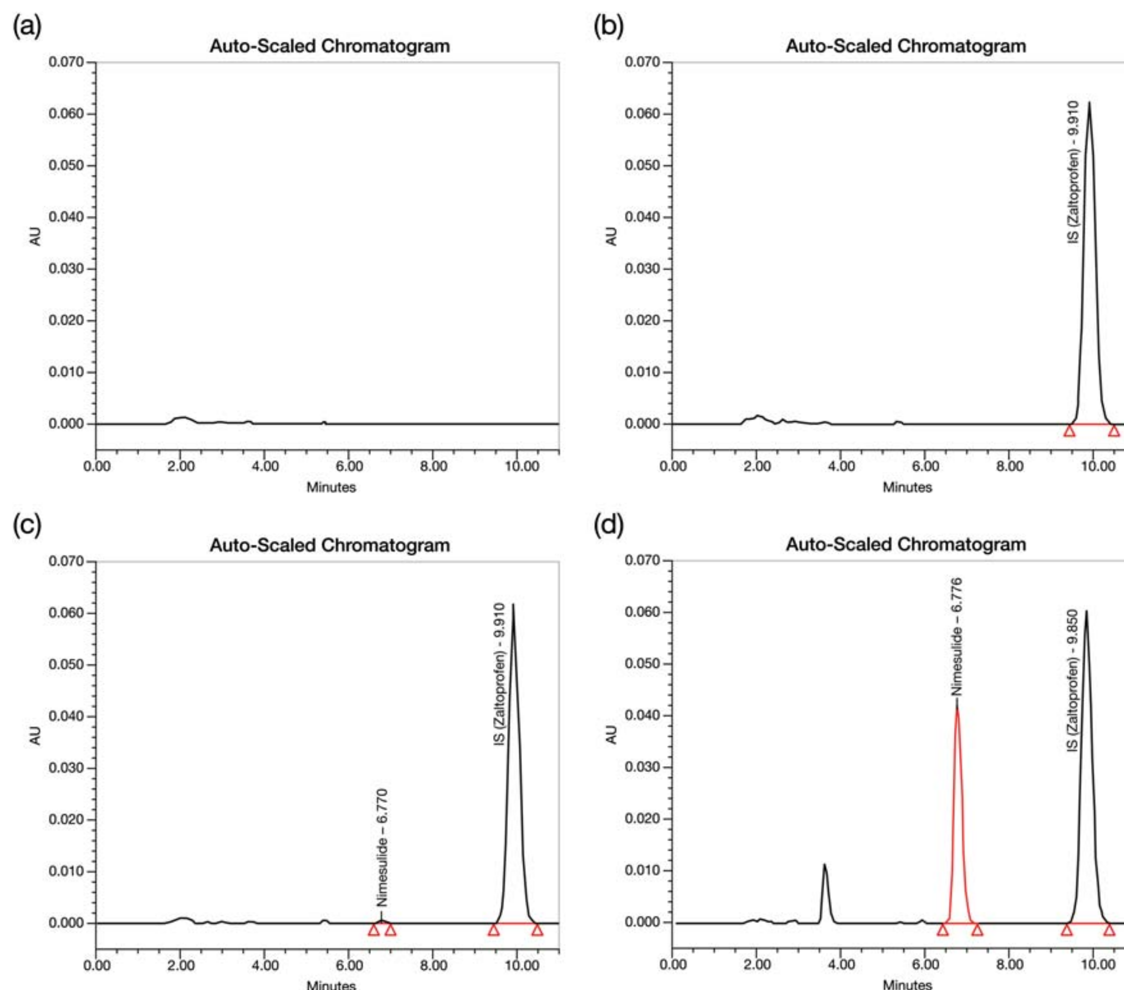

Figure 2. Chromatograms of (a) blank plasma, (b) with IS (300 μg/mL), (c) with nimesulide (LLOQ, 0.2 μg/mL) and IS (300 μg/mL), and (d) human plasma taken 2.0 h after a single oral administration of 2 tablets of 100 mg nimesulide spiked with IS (300 μg/mL).

Table I
Method Validation for the Determination of Nimesulide in Human Plasma and Recovery of Nimesulide and IS (zaltoprofen) after the Extraction Procedure (n = 5)

Nominal concentration (ug/mL, n = 5)	Precision (%R.S.D.)[b]		Accuracy (%)		% Recovery (mean ± SD)	
	Inter-day	Intra-day	Inter-day	Intra-day	Nimesulide	IS
0.2(LLOQ[a])	1.04	5.57	108.50	106.71	102.01 ± 2.30	98.99 ± 1.98
0.6	3.80	0.79	97.96	97.25	98.67 ± 1.71	101.45 ± 3.01
20	4.34	0.71	98.45	98.84	101.98 ± 2.98	99.01 ± 2.78
40	4.45	2.1	98.12	98.14	103.21 ± 2.21	102.36 ± 3.56

[a]LLOQ = Lower limit of quantification, [b]CV = coefficient of variation.

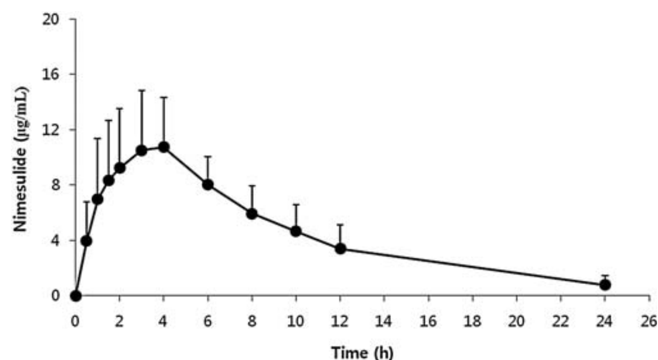

Figure 3. Mean (±S.D.) plasma concentrations vs. time plots after single oral administration of 2 tablets of 100 mg nimesulide to 28 healthy male volunteers.

of two tablets of 100 mg nimesulide in healthy Korean volunteers, C_{max} was 12.06 μg/mL, $AUC_{0-infinity}$ was 113.0 μg-h/mL T_{max} and 3.19 h. Therefore, some pharmacokinetic inconsistency with other reports was ascertained and it is possible that the dosage regimen of nimesulide in the Korean population needs to be changed.

Conclusion

The results indicate that present HPLC-UV method is very simple and sensitive and readily applicable to routine pharmacokinetic and bioavailability studies of nimesulide with reliable analytical results. Therefore, HPLC-UV detection for nimesulide allowed this simple and reliable method to reach a low detection limit of 0.2 μg/mL level and a stable run time within 10.0 min per sample for the pharmacokinetic or bioavailability studies for nimesulide in various laboratory or clinical settings.

Table II
Pharmacokinetic Characteristics after Oral Administration of 2 Tablets of 100 mg Nimesulide in 28 Healthy Subjects

*Parameters	Mean ± SD (n = 28)
$AUC_{0\text{-}24h}$ (μg-h/mL)	106.26 ± 39.65
$AUC_{0\text{-}infinity}$ (μg-h/mL)	113.00 ± 44.85
Extrapolation ($AUC_{24h\text{-}infinity}/AUC_{0\text{-}infinity}$, %)	5.55 ± 4.30
$AUMC_{0\text{-}24h}$ (μg-h^2/mL)	1007.8 ± 220.96
$AUMC_{0\text{-}infinity}$ (μg-h^2/mL)	1021.0 ± 246.48
MRT (h)	8.7 ± 3.37
C_{max} (μg/mL)	12.06 ± 3.05
F/Vd	0.0733
T_{max} (h)	3.19 ± 1.32
$T_{1/2}$ (h)	4.51 ± 1.38
k_a (h^{-1})	0.954 ± 0.056
λ_z (k_e, h^{-1})	0.167 ± 0.047

*AUC = area under plasma concentration-time curve; AUMC = area under first moment of plasma concentration-time curve; MRT = mean residence time; C_{max} = maximal plasma concentration; T_{max} = time for the maximal plasma concentration; $T_{1/2}$ = half-life; k_a = absorption rate constant; $\lambda_z(k_e)$ = elimination rate constant.

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