Determination of Zolmitriptan and its Primary Metabolite, *n*-Desmethy-Zolmitriptan, in Rat Plasma by LC–MS-MS

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The objective of this work was to develop a simple, cost effective, rugged and rapid method for the simultaneous estimation of zolmitriptan (ZP) and its active metabolite *n*-desmethy-zolmitriptan (DZP) in rat plasma to meet the requirement for biological sample analysis. The simple liquid–liquid extraction employed in the present work gave consistent and reproducible recoveries for both the analytes. A mixture of methanol–20 mM ammonium acetate–formic acid (60:40:0.1, v/v/v) was used as mobile phase at a flow rate of 0.3 mL/min. The temperature of column and autosampler were maintained at 40 and 4°C, respectively. The chromatographic run time of each sample was 3.0 min. The limit of quantification was low enough to monitor at least four half-lives of ZP and DZP concentrations with good intra-assay and inter-assay reproducibility for the quality controls. According to the results of all the validation parameters, the method can also be useful for pharmacokinetics study of ZP and for therapeutic drug monitoring in humans with the desired precision and accuracy.

Introduction

Zolmitriptan (ZP) is a new 5-hydroxytryptamine (5-HT) 1B/1D receptor agonist developed for the acute oral treatment of migraine. Clinical studies have shown that oral ZP (2.5 and 5 mg) has an onset of action within 45 min and efficacy is sustained in most patients who respond at 2 h. Head-to-head studies have shown that ZP has efficacy that is similar or superior to sumatriptan in the treatment of migraine (1). At therapeutic doses, ZP has good oral bioavailability in healthy volunteers and has dose-proportional pharmacokinetics that are not affected by food to any clinically relevant extent (2).

ZP inhibits the peripheral trigeminovasular system and is able to access central sites in the brainstem involved in processing cranial pain. Due to high volumes of distribution, ZP occurs at low blood levels after therapeutic administration. It has been reported that in humans, the plasma concentration of ZP was very low (less than 10 ng/mL) when ZP was administered orally in a single 5-mg dose (3). In humans, ZP is rapidly absorbed and undergoes extensive metabolism, during which its primary active metabolite is *n*-desmethy-zolmitriptan (DZP), leading to an increase in the *in vivo* effectiveness of ZP. DZP is another 5-HT 1B/1D receptor agonist. Its activity is 2∼6 times that of ZP and the plasma half-life is longer, so it can prolong the function of abirritation. Because ZP and DZP have significant associations with previously described diseases, the analysis of ZP and DZP in plasma is of great clinical importance.

To perform pharmacokinetic studies, there is a need for analytical methods that quantify ZP and DZP in plasma. Recently, as lower doses have been administrated for ZP, more sensitive liquid chromatography–tandem mass spectrometry (LC–MS-MS) methods have also been developed to obtain full pharmacokinetic profiles (4–6). Vishwanathan (7) reported an LC–MS-MS method to determine four triptans: rizatriptan, ZP, naratriptan and sumatriptan, in human serum, but the lower limit of quantification (LLOQ) of ZP was only 1.0 ng/mL. Zhang (8) established an LC–MS method to analyze ZP in human plasma with an LLOQ of 0.3 ng/mL. Chen (9) reported a high-performance liquid chromatography (HPLC) method with fluorescence detection to analysis ZP in human plasma; the established method could quantify ZP with an LLOQ of 0.2 ng/mL.

However, the authors discovered while searching the PubMed website that simultaneous analysis of ZP and its active metabolite DZP in biological fluids by LC–MS-MS has not been reported. Thus, the aim of the present study was to develop and validate a more sensitive, specific and rapid method for the simultaneous estimation of ZP and its active metabolite DZP in rat plasma by LC–MS-MS. The method should be suitable for routine measurement of biological samples for pharmacokinetic study.

Experimental

Chemicals and reagents

ZP (pKa = 17.14, log P = 1.6) and DZP were purchased from Tlepharmachem Co.(Beijing, China). Rizatriptan (internal standard; IS, pKa = 15.34, log P = 1.4) were purchased from Nal Pharm. The purity of ZP, DZP and IS was >99.3% (as determined by HPLC). Methyl cyanides, methyl *tert*-butyl ether and methanol (HPLC grade) were obtained from Dikma (Shanghai, China). Water was purified by redistillation and filtered through a 0.22-mm membrane filter before use.

Instrument

An Agilent 1100 system consisting of a G1312A quaternary pump, a G1379A vacuum degasser, a G1316A thermostated column oven (Agilent, Waldbronn, Germany) and an HTS PAL auto sampler (CTC Analytics, Switzerland) was used. Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (Applied Biosystems, Toronto, Canada) in multiple reaction monitoring (MRM) mode. A Turbo Ion Spray ionization (ESI) interface in positive ionization mode was used. Data processing was performed with Analyst 1.4.1 software package (Applied Biosystems).

Chromatographic conditions

The chromatographic separation was achieved on a Gemini C18 column (150×2.0 mm, 5 μm; Phenomenex, Torrance, CA) with a Security Guard C18 guard column. A mixture of methanol−20 mM ammonium acetate−formic acid (60:40:0.1, v/v/v) was used as mobile phase at a flow rate of 0.3 mL/min. The temperature of column and autosampler were maintained at 40 and 4°C, respectively. The chromatographic run time of each sample was 3.0 min.

Mass spectrometric conditions

The mass spectrometer was operated using ESI source in the positive ion detection mode. Quantitation was done using MRM mode to monitor protonated precursor → product ion transitions of m/z 288.0 → 58.1 for ZP, 274.0 → 182.1 for DZP and 270.4 → 201.1 for IS (Figure 1).

All parameters for LC and MS were as follows. Turbo spray voltage (IS) was set at 4,500 V. Source temperature was maintained at 550°C. Entrance potential (EP) was set at 6 V. Nitrogen was used as nebulizing gas (10 L/min) and curtain gas (8 L/min). For collision activated dissociation (CAD), nitrogen was employed as the collision gas at a pressure of 4 L/min.

The compound dependent parameters like declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CEP) were optimized at 45, 250, 40 and 10 V for ZP; 50, 200, 40 and 10 V for DZP; and 60, 150, 20 and 10 V for IS, respectively. Quadrupoles 1 and 3 were maintained at unit resolution. Dwell time was set at 200 ms for all the analytes.

Preparation of standard and quality control samples

Stock solutions of ZP, DZP and IS were prepared in methanol at the concentrations of 2, 1 and 2 mg/mL, respectively. The ZP and DZP stock solution was serially diluted with a mixture of methanol−water (50/50, v/v) to provide working standard solutions of the desired concentrations. All solutions were stored at 4°C and brought to room temperature before use. The calibration standards were prepared daily by spiking 100 μL of blank plasma with proper working standard solutions of ZP or DZP and 20 μL internal standard solution (100 ng/mL). The effective concentrations in standard plasma samples were 0.2, 0.5, 1, 2.5, 5, 10, 40 and 50 ng/mL for ZP and DZP. The quality control (QC) samples were prepared in bulk with blank plasma at LLOQ, low (LQC), medium (MQC) and high (HQC) concentrations of 0.2, 0.5, 5 and 40 ng/mL for ZP and DZP, aliquoted and stored at −20°C after preparation. The standards and QCs were extracted on each analysis day with the same procedures for plasma samples as described in the following.

Plasma sample preparation

To a 100-μL aliquot of plasma sample in a 10-mL clean glass tube, 20 μL of IS (100 ng/mL) was added. The samples were vortexed for 1 min and 50 μL of 1 mol/L NaOH + 1.5 mL methyl *tert*-butyl ether was added. The mixture was vortex-mixed for 5 min. After centrifugation at 3,500 rpm for 10 min, the upper organic layer was then transferred into a clean glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in a 100-μL mobile phase, and transferred to an autosampler vial. An aliquot of 10 μL was injected onto the LC−MS-MS system for analysis.

Methodology for validation

A thorough and complete method validation of ZP and DZP in rat plasma was conducted for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, ion suppression/enhancement, cross-specificity, stability and dilution integrity.

Test for selectivity was carried out in six different lots of blank plasma (with heparin sodium as anticoagulant), processed by the same extraction protocol and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and IS.

The linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibration curve ($y = ax + b$, where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$) for ZP and DZP. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the analytes in plasma over the range tested. Intra-batch and inter-batch (on five consecutive days) accuracy and precision were evaluated at three different concentration levels in five replicates for both the analytes. Mean values were obtained for calculated and expressed in terms of percent bias and coefficient of relative standard deviation (%RSD), respectively.

Recovery of the analytes from the extraction procedure was performed at three different concentration levels. Recovery was evaluated by comparing the peak area of extracted samples (spiked before extraction) to the peak area of
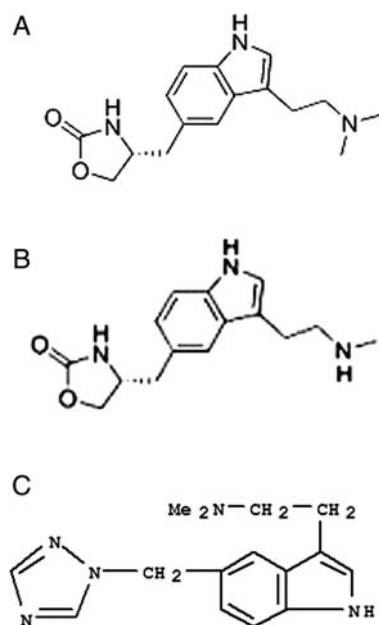

Figure 1. Chemical structures of zolmitriptan (A), N-desmethy-zolmitriptan (B) and rizatriptan (C).

unextracted samples (QC working solutions spiked in extracted plasma).

The matrix effect was evaluated at three concentrations (0.5, 5 and 40 ng/mL in plasma). Two groups of samples were prepared: Group 1 was prepared to evaluate the MS-MS response for a pure standard of ZP and DZP dissolved in the mobile phase (A); Group 2 was prepared in plasma originating from six different donors and submitted to the sample purification process and spiked with ZP and DZP after processing (B). The value (B/A × 100) was considered to be an absolute matrix effect. The inter-subject variability of matrix effect at every concentration level should be less than 15%.

Stability experiments were performed to evaluate the analyte stability in stock solutions and plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed at room temperature and at 4°C by comparing area response of stability sample of analytes and IS with the area response of samples prepared from fresh stock solutions. The results should be within the acceptable limit of ± 10% change for the stock solution stability experiment. Bench-top stability of extracted samples (BTS), room temperature stability (stability in biological matrix, SBM), refrigerated stability at −20°C were performed at three concentrations levels using five replicates at each level. To meet the acceptance criteria, the percent bias should be within ± 15%.

Pharmacokinetic study

Male Sprague-Dawley (SD) rats (weighing 170–220 g) were purchased from the Shanghai institute of Pharmacology and Toxicology and given access to a commercial rat chow diet and tap water. The animals were housed, two per cage, and maintained at $22 \pm 2°C$ and 50–60% relative humidity under a 12:12 h light-dark cycle. The experiments were initiated after acclimation under these conditions for at least one week.

Eight SD rats (both sexes) were assigned oral (p.o.) administration of 0.5 mg/kg ZP. Blood samples were collected immediately before and at 15, 30 and 45 min; and 1, 1.5, 2, 4, 6, 8, 10, 12 and 24 h after drug administration. The blood samples were withdrawn into heparinized Eppendorf tubes and centrifuged at 12,000 rpm for 10 min at 4°C. A 0.2-mL volume of plasma was obtained and stored at −20°C until analysis.

Pharmacokinetic parameters were calculated from the plasma concentration-time data. The elimination half-life ($T_{1/2}$) was determined by linear regression of the terminal portion of the plasma concentration-time data. The area under plasma concentration-time curve from zero to the last measurable plasma concentration point (AUC_{0-t}) was calculated by the linear trapezoidal method. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where C_t is the last measurable plasma concentration and k_e is the terminal elimination rate constant.

Results and Discussion

Method development

As the literature reveals, no report has been made on the simultaneous determination of ZP and DZP in biological fluids by LC−MS-MS. Thus, in the present study, method development was initiated to realize a rugged, sensitive and specific LC−MS-MS method with a short overall analysis time for the simultaneous quantification of ZP and DZP in rat plasma. To accomplish this aim, it was imperative to have a simple, inexpensive and efficient extraction procedure with a short chromatographic run time.

The observed response was much higher in positive ionization mode for all three compounds than in negative mode. Moreover, the major ions observed were m/z 288.0 → 58.1 for ZP, 274.0 → 182.1 for DZP and 270.4 → 201.1 for IS. To develop an accurate, valid and optimal chromatographic condition, different HPLC parameters were all examined and compared, including mobile phase, category of column, column temperature and flow rate of mobile phase. Finally, use of ammonium acetate and formic acid in the mobile phase further enhanced the response for both the analytes and IS with low background noise, resulting in higher sensitivity.

Plasma samples were separated by HPLC on a Gemini C18 column (150×2.0 mm, 5 μm; Phenomenex, Torrance, CA) with a Security Guard C18 guard column. A mixture of methanol−20 mM ammonium acetate−formic acid (60:40:0.1, v/v/v) was used as mobile phase at a flow rate of 0.3 mL/min. The temperature of column and autosampler were maintained at 40 and 4°C, respectively. The representative chromatograms of blank serum, blank serum with added ZP, DZP and IS and sample are shown in Figure 2. The chromatograms show excellent peak shape for both the analytes and the IS. The chromatographic run time of each sample was 3 min. No endogenous interferences were found at the retention times of ZP (1.64 min), DZP (1.63 min) or IS (1.62 min) in the blank plasma. The retention time was short for both analytes, which made it suitable for routine analysis. Under the optimized conditions, the limit of quantification [signal-to-noise (S/N) = 10] of both ZP and DZP was observed to be 0.2 ng/mL.

Linearity, accuracy and precision

The calibration curves for ZP and DZP were linear from 0.2 to 50 ng/mL with correlation coefficients of $r \geq 0.9991$ and 0.9977, respectively, across five regression curves. The equation for mean ($n = 5$) of five calibration curves for the analyte were: ZP, $y = 0.0479x + 0.0075$; DZP, $y = 0.0172x + 0.000268$. The intra-assay precision and accuracy were evaluated in five replicates and are presented in Table I. The table indicated that intra-assay RSDs were between 6.25 and 8.52% for ZP and between 6.12 and 7.95% for DZP. The inter-assay RSDs were between 2.00 and 7.82% for ZP and between 3.66 and 7.84% for DZP.

Recovery and matrix effect

The overall mean recoveries for ZP at LQC, MQC and HQC levels were 84.3, 86.6 and 91.7%, and for DZP were 88.6, 89.2 and 91.5%, respectively, with RSD% between them of 4.3% for ZP and 1.7% for DZP. Thus, the consistency in recoveries of ZP and DZP supported the extraction procedure for its application to routine sample analysis.

The absolute matrix effects for ZP and DZP at concentrations of 0.5, 5 and 40 ng/mL were all within 85−115%, respectively. The absolute matrix effect for IS (40 ng/mL in
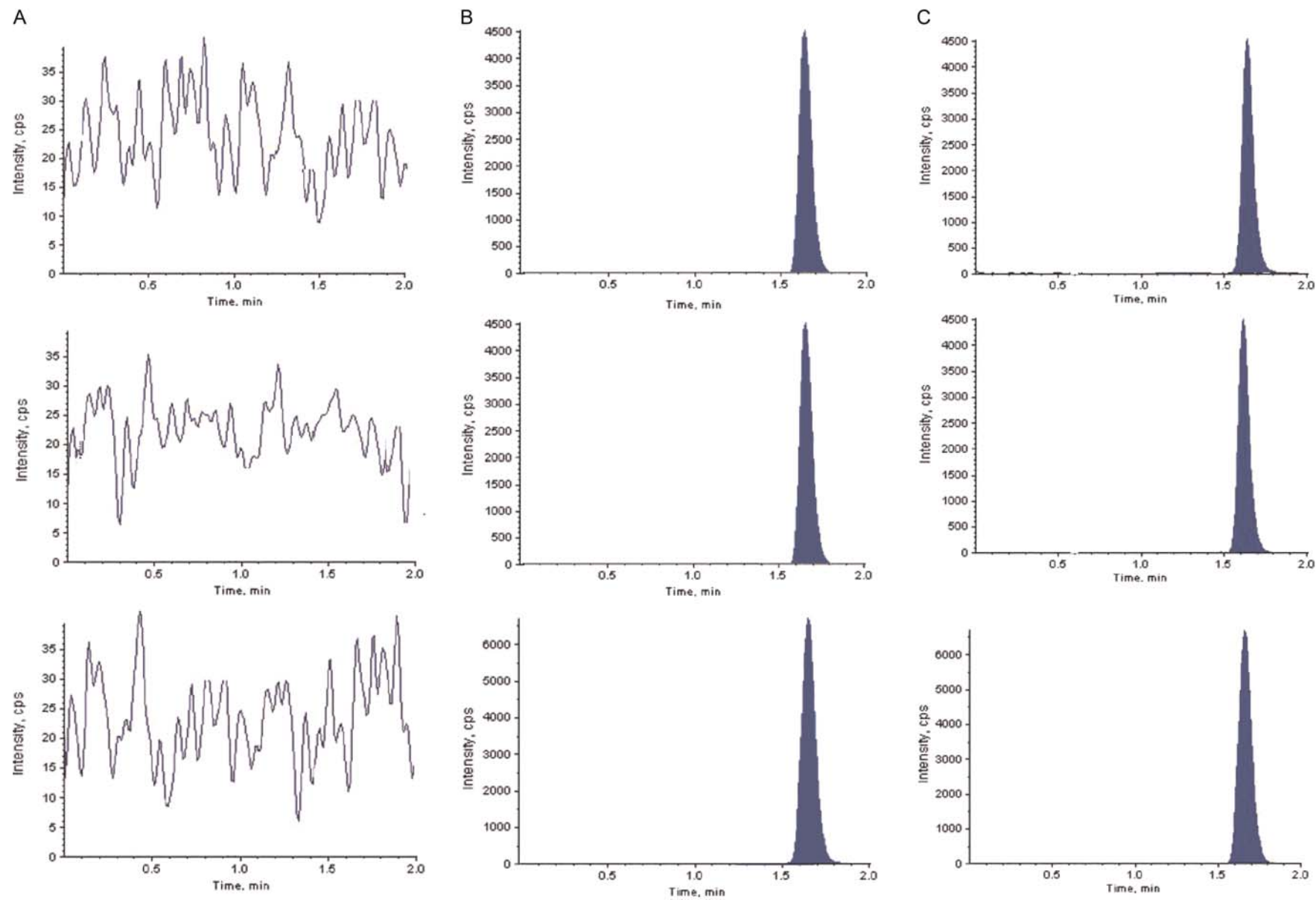
Figure 2. LC—MS–MS chromatograph of ZP: blank serum with added ZP, DZP and IS (A); sample (B).

Table I

Intra-Day and Inter-Day Precision, Accuracy and Extraction Recovery of ZP and DZP Spiked in Plasma by LC−MS-MS ($n = 5$)

Drug	Concentration(ng/ml)	Intra-day (ng/mL)	RSD (%)	RE*(%)	Inter-day(ng/mL)	RSD(%)	RE(%)	Extraction recovery(%)
	0.5	0.48 ± 0.03	6.25	−4.0	0.50 ± 0.01	2.00	0	84.3
ZP	5	5.16 ± 0.44	8.52	3.2	4.96 ± 0.32	6.45	−0.8	86.6
	40	40.12 ± 2.77	6.91	0.3	39.77 ± 3.11	7.82	−0.6	91.7
	0.5	0.49 ± 0.03	6.12	−2.0	0.51 ± 0.04	7.84	2.0	88.6
DZP	5	4.99 ± 0.34	6.81	−0.2	5.11 ± 0.27	5.28	2.2	89.2
	40	39.87 ± 3.17	7.95	−0.3	40.11 ± 1.47	3.66	0.3	91.5

RE: relative error.
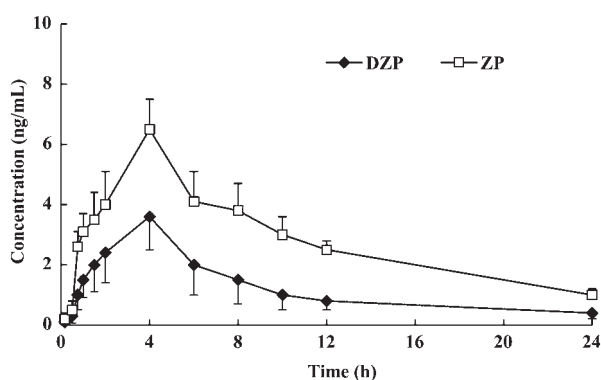
Figure 3. Mean plasma concentration-time profile of ZP after oral administration of 0.5 mg/kg ZP to rats ($n = 5$) (mean ± SD).

Table II

Pharmacokinetic Parameters of ZP and DZP after Oral Administration to Rats (0.5 mg/kg, $n = 5$) (mean ± SD)

Parameters	ZP	DZP
$T_{1/2}$ (h)	3.52 ± 1.31	1.95 ± 0.76
T_{max} (h)	3.66 ± 0.72	3.48 ± 0.68
C_{max} (ng/mL)	6.82 ± 1.78	3.88 ± 0.92
AUC_{0-t} (ng/mL/h)	68.43 ± 14.67	27.38 ± 8.26
$AUC_{0-\infty}$ (ng/mL/h)	76.69 ± 16.22	31.36 ± 7.68

plasma) was 98%. These results showed that ion suppression or enhancement from plasma matrix was negligible under the present conditions.

Stability and dilution integrity

The stability experiments were performed thoroughly to evaluate their stability in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was compared with freshly prepared QC samples. Stock solutions of ZP, DZP and IS were stable at room temperature for 12 h and at 4°C for 30 days for ZP, DZP and IS, with mean percent change well within 7.5%. The intermediate solution of ZP, DZP and IS in methanol−water (50:50, v/v) was stable for 30 days. Both analytes were found to be stable in controlled plasma at room temperature up to 24 h and for at least three freeze and thaw cycles. The analytes in extracted plasma samples were stable for 24 h under refrigerated conditions at 4°C. Bench-top stability of extracted samples was also found up to 24 h. The ZP and DZP spiked plasma samples stored at −20°C for long-term stability were found to be stable for minimum period of 30 days.

Application

This newly developed method was applied to determine the plasma concentration of ZP and DZP in rats following oral (0.5 mg/kg) administrations. The mean plasma concentration-time profiles of ZP and DZP after oral administration is illustrated in Figure 3 and the estimated pharmacokinetic parameters are presented in Table II. ZP was rapidly absorbed into the circulation system and reached its peak concentration at approximately 4 h after oral administration, then rapidly metabolized to the primary metabolite DZP. The major pharmacokinetic parameters of ZP and DZP were as follows: C_{max}, 6.82 ± 1.78 and 3.88 ± 0.92 ng/mL; $T_{1/2}$, 3.52 ± 1.31 h and 1.95 ± 0.76; T_{max}, 3.66 ± 0.72 and 3.48 ± 0.68; AUC_{0-t},e 68.43 ± 14.67 and 27.38 ± 8.26 ng/mL/h; $AUC_{0-\infty}$, 76.69 ± 16.22 and 31.36 ± 7.68 ng/mL/h. The results regarding C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t} and $AUC_{0-\infty}$ of ZP agree with the present report. Therefore, the pharmacokinetic parameters of DZP using a rat model was credible. In addition, as we know, there are three primary metabolites from ZP: indole acetic acid, N-oxides and N-demethyl metabolites (DZP), of which only DZP is active. Pharmacokinetic curve shows that the plasma concentrations of DZP are roughly half of the parent drugs. Therefore, it was estimated that the metabolites may enhance the efficacy of ZP through the determination.

Conclusion

The objective of this work was to develop a simple, cost effective, rugged and rapid method for the simultaneous estimation of ZP and its active metabolite DZP in rat plasma to meet the requirement for biological sample analysis. The simple liquid−liquid extraction employed in the present work gave consistent and reproducible recoveries for both the analytes. The limit of quantification was low enough to monitor at least four half-lives of ZP and DZP concentration with good intra-assay and inter-assay reproducibility for the quality controls. According to the results of all the validation parameters, the method also can be useful for pharmacokinetics study of ZP and for therapeutic drug monitoring in humans with desired precision and accuracy.

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