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A Validated Bioanalytical Method for Quantification of Ziprasidone in Rabbit Plasma by LC-MS/MS: Application to a Pharmacokinetic Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author VMB designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors KB and BSRC, RN managed the literature searches, Author KB analyses of the study performed the spectroscopy analysis managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

A Liquid-liquid extraction method was developed for quantification of Ziprasidone in Rabbit plasma by a suitable bio-analytical method with LC-MS/MS. Ziprasidone d8 was used as an internal standard for Ziprasidone. Hypurity C18, 150 x 4.6 mm, 5 µm column used for chromatographic separation of analyte followed by detection with mass spectrometry. The total run time was 4.0 minutes. The proposed method has been validated with the linear range of 0.05 – 200.00 ng/mL for Ziprasidone. The intra-run and inter-run precision values were within 0.625 to 0.947% and 2.182 to 3.198% for Ziprasidone. The overall recovery for Ziprasidone and Ziprasidone d8 was 92.57% and 95.70% respectively. This validated method was successfully applied into the pharmacokinetic study of Rabbit plasma.

Keywords: Ziprasidone; Rabbit plasma; mass spectrometry; pharmacokinetic.

1. INTRODUCTION

Ziprasidone is a psychotropic agent belonging to the chemical class of benzisoxazole derivatives and is indicated for the treatment of schizophrenia. Ziprasidone is a selective monoaminergic antagonist with high affinity for the serotonin Type 2 (5HT2), dopamine Type 2 (D2), 1 and 2 adrenergic, and H1 histaminergic receptors. The chemical name for ziprasidone is 5-[2-[4-(1,2-benzisothiazol-3-yl)-1-piperazinyl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one. Its molecular formula is $C_{21}H_{21}ClN_4OS$ and its molecular weight is 412.94. [1]

Literature survey reveals that, there are few methods reported for quantification of Ziprasidone by dielectric spectroscopy (2), HPLC (3-11), UPLC (12-13), LC-MS (14-20). Among all, few methods were developed in Pharmaceutical [2-8, 12]. Biological (9-11, 13-20) samples. Among all LC-MS/MS (14-20) methods were achieved best results in terms of clinical pharmacokinetic and bioequivalence studies. Few methods were reported for quantification of Ziprasidone in rat brain (19) and rat plasma (20) by LC-MS for preclinical pharmacokinetic study. There is no method was reported for quantification of Ziprasidone in rabbit plasma by LC-MS/MS for preclinical pharmacokinetic study.

The aim of the present research includes development and validation of the method for quantification of Ziprasidone in rabbit plasma by using LC-MS/MS with short run time and by using small volume of plasma sample.

2. EXPERIMENTAL

2.1 Chemicals and Reagents

Ziprasidone (*ZI*) (98.90% purity) was purchased from USP, and Ziprasidone d8 (*ZIIS*) (97.50% purity) was obtained from Creative Organics, India. Zispradone and Zispradone d8 structure has been shown in Fig. 1.

HPLC grade methanol and acetonitrile were purchased from Jt. Baker Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA. Formic acid was purchased from Merck Limited, worli, Mumbai., Ammonium acetate, Sodium carbonate (reagent grade) were purchased from Merck Limited, worli, Mumbai. Methyl t-butyl ether was purchased from RCI Labscan, Mumbai. Rats were obtained from Anthem biosciences, Bangalore, India. Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) was used in the study. All other chemicals in this study were of analytical grade.

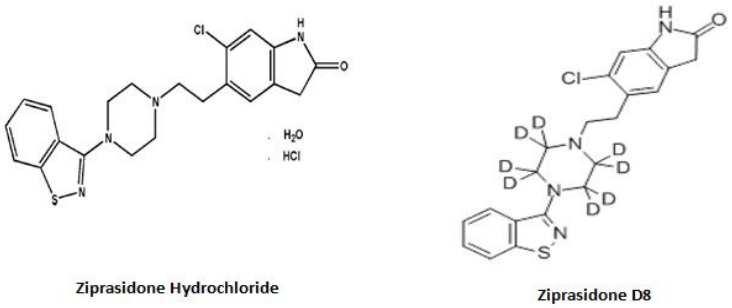

Fig. 1. Chemical structures of ziprasidone and ziprasidone D8

2.2 Instrumentation

HPLC system (1200 series model, Agilent Technologies, Waldbronn, Germany), Mass spectrometry API 4200 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) multiple reaction monitoring (MRM) with possitive ionization mode was used. Data processing was performed on Analyst 1.5.1 software package (SCIEX).

2.3 Detection

The mass spectrometer was operated in the multiple reaction monitoring (MRM) with electro spray ionisation (ESI) in the possitive ion mode. Source dependent parameters optimized were as nebulizer gas flow: 5 psi; CAD Gas 5 psi; curtain gas flow: 25 psi; temperature (TEM): 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 40, 20, 10, 22, 12 eV for both Ziprasidone and Ziprasidone d8. Quadrupole 1 and quadrupole 3 were both maintained at a unit resolution and dwell time was set at 200 ms for both Ziprasidone and Ziprasidone d8. The mass transitions were selected as m/z 413.2 → 194.0 for Ziprasidone and m/z 421.2→ 194.0 for Ziprasidone d8. The parent and product ion spectra for Ziprasidone and Ziprasidone d8 were represented in Fig. 2a-2d.

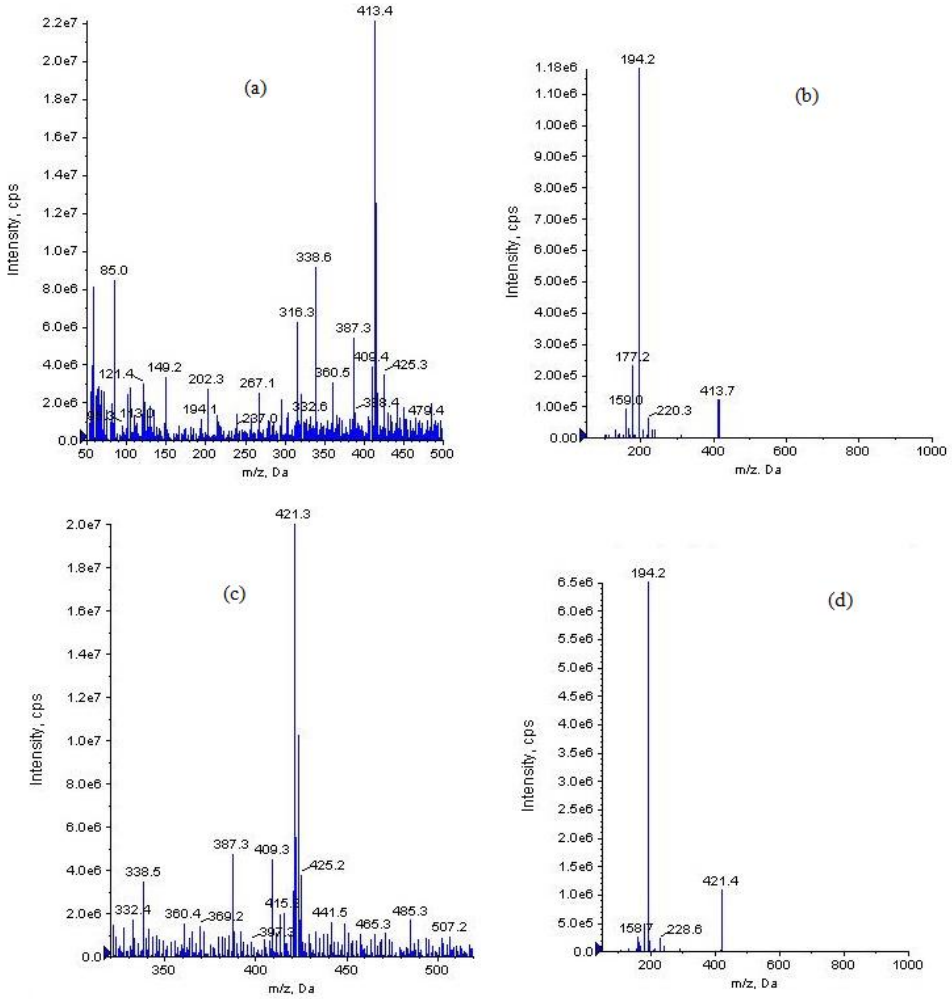

Fig. 2. Mass spectrum of (a) ziprasidone parention, (b) ziprasidone production, (c) ziprasidone d8 parention, (d) ziprasidone d8 production

2.4 Chromatography

Hypurity C18, 150 x 4.6 mm, 5 µm was selected as the analytical column. Column temperature was set at 40°C. Mobile phase composition was 2 mM Ammonium acetate: Acetonitrile (5:95, v/v). Source flow rate was 1000 µL/min without split with injection volume of 5 µL. Ziprasidone , Ziprasidone d8 were eluted at 2.3 ± 0.2 min and 2.3 ± 0.2 min respectively, with a total run time of 4.0 min for each sample.

2.5 Calibration Curve and Quality Control Samples

Two separate stock solutions of Ziprasidone were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as sample analysis. The stock solutions of Ziprasidone and Ziprasidone d8 were prepared in 60% methanol in 0.1% formic acid at free base concentration of 100.00 µg/mL. Primary dilutions and working solutions were prepared from stock solutions using blank plasma. These working solutions were used to prepare the calibration curve and quality control samples. Blank Rabbit plasma was screened prior to spiking to ensure, free of endogenous interference at retention time of Ziprasidone and Ziprasidone d8. Ten point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Ziprasidone. Calibration samples were made at concentrations of 0.05, 0.10, 0.20, 0.40, 2.00, 20.0, 40.00, 80.00, 160.00 and 200.00 ng/mL and quality control samples were made at concentrations of 0.05, 0.15, 100.00, and 140.00 ng/mL for Ziprasidone. All the calibration and quality control samples were stored at -30°C until analysis.

2.6 Sample Preparation

Liquid-Liquid extraction procedure was used in this study for extraction of Ziprasidone from the plasma samples. For this purpose, 50 µL of Ziprasidone d8 (50.00 ng/ mL), 50 µL plasma (respective concentration of plasma sample), 100 µL of sodium carbonate solution was added into ria vials then vortexed for 30 seconds followed by 2.5 mL of methyl t-butyl ether was added and vortex for 5 minutes. Then samples were centrifuged at 4000 rpm for approximately 5 min at ambient temperature and transfered the supernatant from each sample into respective Ria vials and evaporated to dryness and reconstituted with 400 µL of 20 mM Ammonium acetate: Acetonitrile (5:95, v/v) followed by vortexed briefly. Finally sample was transfered into auto sampler vials to inject into LC-MS/MS.

2.7 Selectivity

Selectivity was performed by analyzing the six different rabbit blank plasma samples to test for interference at the retention time of analyte.

2.8 Matrix Effect

Matrix effect for Ziprasidone and Ziprasidone d8 was evaluated by comparing peak area ratio in post-extracted plasma sample from 6 different drug-free blank plasma samples and aqueous reconstitution samples. Experiments were performed at LQC and HQC levels in triplicate with six different plasma lots with the acceptable precision (%CV) of ≤ 15%.

2.9 Precision and Accuracy

It was determined by replicate analysis of quality control samples (n = 6) at lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

2.10 Recovery

The extraction efficiencies of Ziprasidone and Ziprasidone d8 were determined by analysis of six replicates at each quality control concentration level for Ziprasidone and at one concentration for the Ziprasidone d8. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak area of non extracted standards.

2.11 Stability

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. The stability of spiked Rabbit plasma samples stored

at room temperature (bench top stability) was evaluated for 24 h. The stability of spiked Rabbit plasma samples stored at 2-8°C in autosampler (autosampler stability), and it was evaluated for 26 h. The reinjection reproducibility stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 2-8°C for 24 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze–thaw stability evaluation. For long term stability evaluation the concentrations obtained after 55 days were compared with initial concentrations.

2.12 Application of Method

The validated method has been successfully used to analyze Ziprasidone concentrations (1.6mg/1.8kg, oral route) in 6 Rabbits. The study was conducted according to current GCP guidelines. Before conducting the study it was approved by an authorized animal ethics committee (Institutional Animal ethics committee) of albino research and training institute,miyapur, Hyderabad. There were a total of 13 blood collection time points including the pre-dose sample. The blood samples were collected in separate vacutainers containing K_2EDTA as an anticoagulant. The plasma from these samples was separated by centrifugation at 4000 rpm within the range of 20°C. The plasma samples were stored at -30°C till analysis. The pharmacokinetic parameters were computed using WinNonlin® software version 5.2.

3. RESULTS AND DISCUSSION

3.1 Method Development

During method development, different options were evaluated to optimize mass spectrometry detection parameters, chromatography and sample extraction.

3.1.1 Mass spectrometry detection parameters optimization

Electro spray ionization (ESI) provided a maximum response over a atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to ion source operated at both polarities at a flow rate of 1.0 mL/min. Ziprasidone gave more response in positive ion mode as compare to the negative ion mode. The predominant peaks of Ziprasidone and Ziprasidone d8 correspond to the [M+H]+ ions at m/z 413.2 and 421.2 respectively. [Fig. 2a, Fig. 2c]. Product ions of Ziprasidone and Ziprasidone d8 scanned in quadrupole at m/z of 194.0 and 194.0 respectively. [Fig. 2b, Fig. 2d].

3.1.2 Chromatography optimization

Initially, a mobile phase consisting of ammonium acetate and methanol with different combinations were tried, but a low response was observed. The mobile phase containing acetic acid: acetonitrile (20:80, v/v) and acetic acid: methanol (20:80, v/v) gave the better response, but poor peak shape was observed. A mobile phase of ammonium acetate and acetonitrile with different combinations were tried. Using a mobile phase containing ammonium acetate and acetonitrile 95:5, v/v), the best signal along with a marked improvement in the peak shape was observed for Ziprasidone and Ziprasidone d8. Short length columns, such as symmetry shield RP18 (50 mm x 2.1 mm, 3.5 µm), inertsil ODS-2V (50 mm x 4.6 mm, 5 µm), hypurity C18 (150 mm x 4.6 mm, 5 µm) and hypurity advance (50 mm x 4.0 mm, 5 µm) YMC basic (50 mm x2 mm, 5 µm), inert sustain C18, HP 3 µm, 4.6 x 50 mm, were tried during the method development. The best signal and good peak shape was obtained using the Hypurity C18, 150 x 4.6 mm, 5 µm, column. It gave satisfactory peak shape for both Ziprasidone and Ziprasidone d8. Flow rate of 1.0 mL/min without splitter was used and reduced the run time to 4.0 min. Both drug and internal standard were eluted with shorter time at 2.3 min. For an LC-MS/MS analysis, utilization of stable isotope-labeled or suitable analog drugs as an internal standard proves helpful when a significant matrix effect is possible. However, in our case, Ziprasidone d8 was found to be best for the present purpose in LLE extraction method. The column oven temperature was kept at a constant temperature of about 40°C. Injection volume of 5µL sample is adjusted for better ionization and chromatography.

3.1.3 Extraction optimization

Prior to load the sample for LC injection, the co-extracted proteins should be removed from the prepared solution. For this purpose, initially

tested with different extraction procedures like Protein precipitation (PPT), Liquid- liquid extraction (LLE) and solid phase extraction (SPE). It was found that ion suppression effect in protein precipitation method for drug and internal standard. Further, tried with SPE and LLE. Out of all, we observed LLE is suitable for extraction of drug and IS. There was no significant effect of IS on analyte recovery, sensitivity, ion suppression or ion enhancement. High recovery and selectivity was observed in the Liquid-liquid extraction method.

These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of Ziprasidone in Rabbit plasma.

3.2 Method Validation

A thorough and complete method validation of Ziprasidone in Rabbit plasma was done following FDA bio-analytical method validation guideline [21]. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, reinjection reproducibility and stability.

3.2.1 Selectivity and sensitivity

Representative chromatograms obtained from blank plasma and plasma spiked with a lower limit of quantification (LOQ), upper limit of quantification (ULQ) sample were shown in Fig.3. for Ziprasidone and Ziprasidone d8. The mean % interference observed at the retention time of analyte between six different lots of Rabbit plasma, containing K_2EDTA as an anti-coagulant calculated for Ziprasidone and Ziprasidone d8 respectively, which was within acceptance criteria. The LOQ for Ziprasidone was 0.05 ng/mL. All the values obtained below 0.05 ng/mL for Ziprasidone were excluded from statistical analysis as they were below the LOQ values validated for Ziprasidone.

3.2.2 Matrix effect

The CV % of ion suppression/enhancement in the signal was found as 0.35% and 0.73% at LQC and HQC level for Ziprasidone, indicating that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

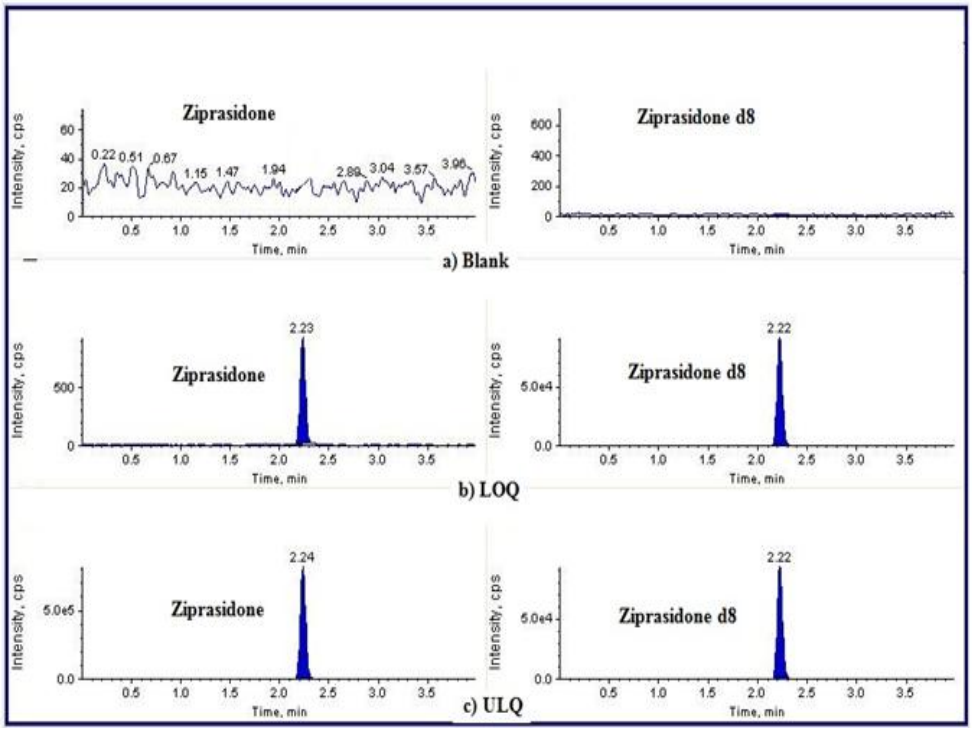

Fig. 3. Chromatogram of a) Blank b) LOQ c) ULQ of ziprasidone and ziprasidone d8

3.2.3 Linearity

The peak area ratios of calibration standards were proportional to the concentration of Ziprasidone in each assay over the nominal concentration range of 0.05-200.00 ng/mL. The calibration curves appeared linear and were well described by least-squares quadratic regression lines. As compared to the 1/x weighing factor, a weighing factor of 1/x2 properly achieved the best result and was chosen to achieve homogeneity of variance. The correlation coefficient was ≥0.9850 for Ziprasidone. The observed mean back-calculated concentration with accuracy and precision (% CV) of five linearity's analyzed during method validation is given in Table 1.

The deviations of the back calculated values from the nominal standard concentrations were less than 15%. This validated linearity range justifies the concentration observed during real sample analysis.

3.2.4 Precision and accuracy

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 6) quality control over five separate batch runs analyzed on four different days. The inter-run, intra-run precision (% CV) was ≤15% and inter-run, intra-run accuracy was in between 85-115% for Ziprasidone. All these data presented in Table 2 indicate that the method is precise and accurate.

3.2.5 Recovery

Six aqueous replicates (samples spiked in reconstitution solution) at low, medium and high quality control concentration levels for Ziprasidone were prepared for recovery determination, and the areas obtained were compared versus the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Ziprasidone was 92.57% with a precision of 4.40%, and the mean recovery for Ziprasidone d8 was 95.70% with a precision of 3.1%. This indicates that the extraction efficiency for the Ziprasidone as well as Ziprasidone d8 was consistent and reproducible.

3.2.6 Reinjection reproducibility

Reinjection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during sample analysis. The change was less than 1.2% for LQC and HQC level concentration; hence batch can be reinjected in the case of instrument failure during sample analysis. Furthermore, samples were prepared to be reinjected after 24 hours, which shows % change less than 1.2% for LQC and HQC level concentration; hence batch can be reinjected after 24 hours in the case of instrument failure during real subject sample analysis.

3.2.7 Stabilities

Stock solution stability was performed to check stability of Ziprasidone and Ziprasidone d8 in stock solutions prepared in 60% methanol in 0.1% formic acid and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 15 days. The % change for Ziprasidone and Ziprasidone d8 were 0.23% and 0.14% respectively indicate that stock solutions were

Table 1. Calibration curve details

Spiked plasma concentration (ng/mL)	Concentration measured (mean) (ng/mL)	SD	(%) CV (n = 5)	Accuracy %
0.05	0.05	0.00	0.87	100.00
0.10	0.10	0.00	0.89	100.75
0.20	0.19	0.01	8.70	96.16
0.40	0.40	0.00	0.98	102.25
2.00	2.01	0.07	3.56	100.51
20.00	19.68	0.49	2.49	98.44
40.00	40.18	0.60	1.50	100.47
80.00	81.46	1.69	2.08	101.82
160.00	162.66	2.11	1.30	101.66
200.00	195.70	5.26	2.68	97.85

stable at least for 15 days. Room temperature and auto sampler stability for Ziprasidone was investigated at LQC and HQC levels. The results revealed that Ziprasidone was stable in plasma for at least 26 h in an auto sampler at 2-8°C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Ziprasidone at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Ziprasidone was stable in a matrix up to 55 days at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in Table 3.

3.3 Application

The validated method has been successfully applied to quantify Ziprasidone concentrations into a single dose (1.6 mg/1.8 kg) in rabbit. Rabbits were obtained from albino research and training institute, miyapur, Hyderabad. After oral administration of drug 0.3 ml of blood samples for analytical determinations were collected via the ear nerves at specific time intervals for 30 h. Plasma samples were stored at −30ºC until analysis. The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-30}(area under the plasma concentration–time curve measured 30 hours, using the trapezoidal rule), T_{max} (time to observe maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression) and $T_{1/2}$ (terminal half-life as determined by quotient $0.693/K_{el}$) [22,23]. Pharmacokinetic details were shown in Table 4. The mean concentration versus time profile of Ziprasidone in Rabbit plasma is shown in Fig. 4.

Table 4. Pharmacokinetic parameters of ziprasidone in rabbit plasma

Pharmacokinetic parameter	values
AUC_{0-t} (ng · h/mL)	215.37
C_{max} (ng/ mL)	26.91
$AUC_{0-\infty}$ (ng · h/mL)	229.04
Kel	0.08627
T_{max} (h)	3
$t_{1/2}$	8.03

$AUC_{0—\infty}$: area under the curve extrapolated to infinity, $AUC_{0—t}$: area under the curve up to the last sampling time, Cmax: the maximum plasma concentration, Tmax: the time to reach peak concentration, Kel: the apparent elimination rate constant

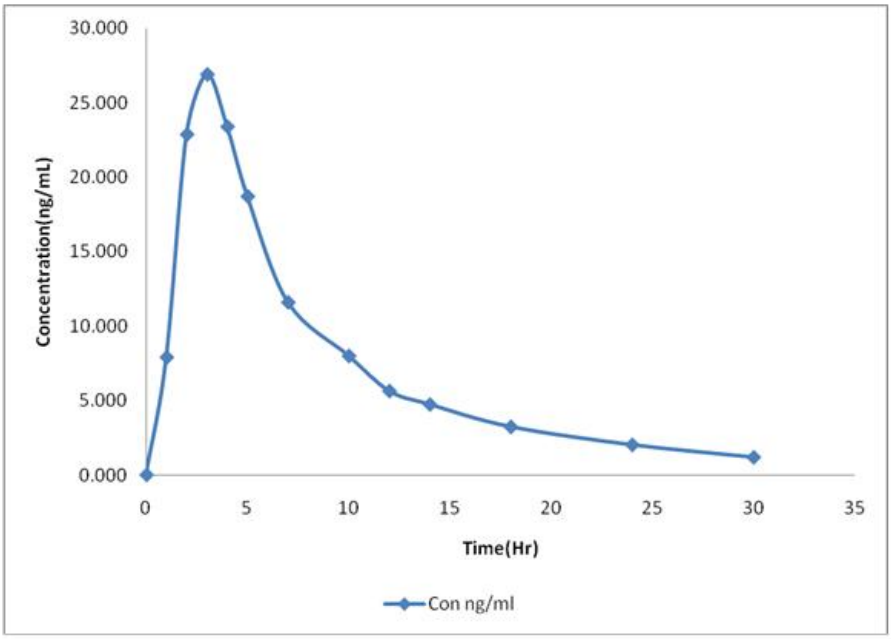

Fig. 4. Mean plasma concentrations vs. time graph of ziprasidone after oral administration of 1.6 mg/1.8 kg male rabbit

Table 2. Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

Spiked plasma concentration (ng/mL)	Intra-run			Inter-run		
	Concentration measured (*n*=6) (ng/mL) (mean ± S.D)	(%) CV	% Accuracy	Concentration measured (*n*=30) (ng/mL) (mean±S.D.)	(%) CV	% Accuracy
0.05	0.05±0.00	7.25	108.16	0.05±0.00	6.15	108.16
0.15	0.15±0.00	2.86	100.83	0.15±0.00	4.32	103.83
100.00	100.33±3.96	3.95	100.33	102.64±2.59	2.52	102.64
140.00	136.96±5.04	3.68	97.83	140.49±6.18	4.40	100.35

Table 3. Stability of the samples

Spiked plasma concentration (ng/mL)	Room temperature stability		Autosampler stability		Long term stability		Freeze and thaw stability	
	24.0 h		26 h		55 days		Cycle 3 (48 h)	
	Concentration measured (n=6) (ng/ mL)(mean ±S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL)(mean±S.D)	% CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean±S.D)	%CV (*n*=6)
0.15	0.15±0.00	4.44	0.14±0.00	0.675	0.15±0.00	3.46	0.15±0.00	3.19
140.00	140.19±1.48	1.06	138.15±0.64	0.469	140.15±3.05	2.18	140.15±3.05	2.18

4. CONCLUSION

The proposed bio-analytical method is most specific, highly sensitive, rugged and reproducible. The major advantage of this method is rapid analysis time (4.0 min), less plasma volume (50 µl) usage for analysis. This method was successfully applied in pharmacokinetic study to evaluate the plasma concentrations of Ziprasidone in healthy male rabbits.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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