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SENSITIVE, HIGH-THROUGHPUT AND ENANTIOSELECTIVE QUANTITATION OF ZOPICLONE IN HUMAN PLASMA BY LCMS/MS: APPLICATION TO A HUMAN PHARMACOKINETIC STUDY

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ABSTRACT: Zopiclone (ZPC), a cyclopyrrolone derivative, is a short-acting hypnotic agent. Its pharmacological properties include hypnotic, sedative, anxiolytic, anticonvulsant and muscle-relaxant actions. ZPC has a single chiral centre, and its enantiomers are S-zopiclone (S-ZPC) and R-zopiclone (R-ZPC). S-ZPC was adjudge to possess two- fold enhanced pharmacological activity than racemate (ZPC) while its opposite enantiomer R-ZPC appears to have almost zero activity and elevated toxicity. A high performance LC-MS\MS method employing positive electrospray ionization was developed for the simultaneous determination of both S-ZPC and R-ZPC in human plasma samples by using S-Zopiclone d8 as internal standard. Analytes were extracted from plasma samples by employing solid phase extraction technique. After extraction, 5µL of clean sample was injected on to chromatographic system and distinct separation achieved on chiralpak IC-3, 0.46 cm I.D*15 cm L column with the isocratic mobile phase consisting of a mixture of Ammonia in Acetonitrile and Milli Q Water. Detection was performed using API 4000 MS that monitors specific transition per compound in Multi Reaction monitoring mode. Limit of quantitation achived is 0.500ng/mL. The developed method is validated as per USFDA guidelines and used for quantifying S-ZPC and R-ZPC in human plasma and has been successfully applied to a pharmacokinetic study.

INTRODUCTION: Zopiclone belongs to the group of medicines called central nervous system (CNS) depressants. The therapeutic pharmacological properties of Zopiclone include hypnotic, anxiolytic, anticonvulsant, and myorelaxant properties.

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Chemically Zopiclone is (5RS)-6-(5-Chloropyridin-2-yl)-7-oxo-6, 7-dihydro -5H-pyrrolo [3, 4-b] pyrazin-5-yl 4-methyl piperazine-1-carboxylate [1].

Zopiclone has a single chiral centre. Zopiclone, a cyclopyrrolone, is a non-benzodiazepine derivative that binds at the BZD-ionophore: chloride channel complex. It's absorption time is approximately 2 hours with a bioavailability of 70% and the elimination half-life is 5 hours. Renal excretion occurs after enterohepatic cycling [2].

Zopiclone contain chiral centres, and (S)-zopiclone is active dextrorotatory stereoisomer of Zopiclone[3] and binding at benzodiazepine recognition sites is about 50-fold higher than that of (R)-zopiclone [4], But pharmacokinetics of enantiomers varies between individuals [5]. Zopiclone prolongs non-REM (rapid eye movement) stage 2 and 4 sleep with a significant decrease in total REM sleep. Zopiclone also reduces stage 1 sleep and increases stage 3 sleep. Zopiclone was the first compound developed which is chemically unrelated to benzodiazepines yet binds with high affinity to benzodiazepine receptors [6].

Zopiclone is most frequently prescribed hypnotics in Europe [7] which, at usual therapeutic doses, decreases sleep latency and the number of nocturnal awakenings, increases total sleep time and improves sleep quality [8].

Several papers have been published for the quantitation of Zopiclone in biological fluids using HPLC–UV [9], GC–MS [10] and LC/MS/MS techniques [11-15]. Further, papers are published on S-enantiomer of Zopiclone i.e., Eszopiclone quantitation by LC-MS/MS methods in biological fluids [16-17]with HPLC [18-19] were reported.

ZPC enantiomers have been analyzed using a Chiralcel OD-H column, ethanol–hexane (60:40, v/v) as the mobile phase and fluorescence detection [20]. Further ZPC steroselectivity distribution and conversion in rat tissues also reported [21].

There are other methods in which Zopiclone was estimated by LC-MS\MS for the establishment of blood stability and degradation in whole blood at different storage conditions in forensic departments [22-24]. Later ZPC and its enantiomers were estimated with RP-HPLC [25-26] in bulk drug and tablet dosage forms were also reported.

Till now, only one LC-MS/MS method has been reported for the Enantioselective analysis of Zopiclone and its metabolites in plasma by liquid chromatography/tandem mass spectrometry [27]. The LC system was operated under isocratic mode with the mobile phase consisting of ethanol–methanol–acetonitrile (50:45:5, v/v/v) plus 0.025% diethylamine, pumped at a flow-rate of 1.0 mL min^{-1}.

A post-column infusion of 1% acetic acid aqueous solution was delivered at a flow-rate of 0.25 mL min^{-1} to improve the MS detection. The HPLC eluent was split by a Valco zero-dead-volume internal Tee (Restek, Pennsylvania, PA, USA) and a flow-rate of approximately 0.2 mL min^{-1} was introduced into the stainless steel capillary probe. The run time of this method is ~18minutes.

ZPC and its metabolites were isolated from aliquots of 0.5 mL of rat plasma samples by liquid–liquid extraction. The developed method was linear over the concentration range of 7.5–500 ng mL^{-1}. The validated method was employed in a study of kinetic disposition of ZPC and its metabolites in rats.

The objective of the present work was to develop a sensitive, high throughput, simple and enantioselective method for the simultaneous quantification of S and R enantiomers of Zopiclone in human plasma samples by LC-MS/MS. The validated method was applied in an enantioselective bioequivalence study of Zopiclone. This method offers various advantages over other published methods, such as;

(A) It requires less sample volume (200µL plasma), hence subjects are required to bleed less,

(B) High throughput (~65% reduction in runtime), hence, greater number of samples can be analyzed in a day,

(C) Sensitivity: ~15 times more sensitive than previously reported methods,

(D) Simplicity (use of single column without complex sequential techniques like column switching or post column infusion).

MATERIALS AND METHODS:

Chemicals and reagents: Working standards of S-Zopiclone (S-ZPC, **Figure 1**), R-Zopiclone (R-ZPC, Figure 1) and S-Zopiclone-D8 (S-ZPC D8, Figure 1) were procured from Clearsynth Labs (P) Ltd., Mumbai, India. Purity of both working standard was found to be >95%. HPLC grade acetonitrile and methanol were purchased from J.T. Baker (USA). MS grade Formic Acid was purchased from Sigma Aldrich (Fluka). MS grade Ammonia was purchased from Qualigens.

De-ionized water was produced from Milli-Q Gradient A-10 system (Millipore, MA, USA). EDTA blank human plasma was collected from Clinical Pharmacology Unit of Reliance Life Sciences Private Limited, Navi Mumbai, India through Ethics Committee approved informed consent process in compliance with ICH-GCP.

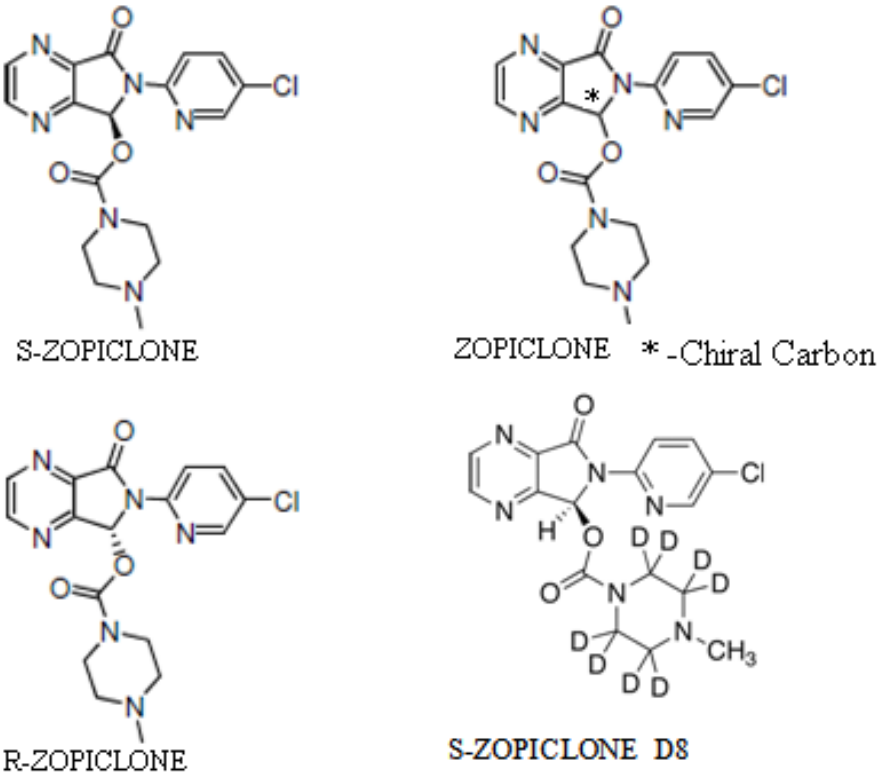


FIGURE 1: STRUCTURAL REPRESENTATION OF ANALYTES AND INTERNAL STANDARD

HPLC Operating Conditions: Shimadzu (Shimadzu Scientific Instruments, Columbia, U.S.A.) Prominence LC system equipped with degasser (DGU-20A_3), pumps (LC-20AD) along with auto-sampler (SIL HTc) were used. 5µL aliquots of the processed samples were injected on a Chiralpak IC-3, 0.46 cm I.D*15 cm L (Daicel Chiral Technologies Pvt. Ltd., India) operated at 25°C. The isocratic mobile phase, a mixture of 0.1% Ammonia in Acetonitrile and 0.1% Ammonia in Milli Q Water (90:10 v/v) was delivered at a flow rate of 1000µL/min with a three way splitter into electro spray ionization chamber of Mass spectrometer with a total run time of 7.00 mins.

Mass Spectrometry operating conditions: Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using an AB Sciex (Foster City, CA, USA) API 4000 mass spectrometer, equipped with a Turboionspray™ interface at 450°C. The Optimized Mass Spectrometric conditions were mentioned in **Table 1** and Quadrupole Q1 and Q3 were set on unit resolution mode. Analytical data was processed using Analyst software (version 1.4.1).

TABLE 1: MS CONDITIONS

Drug Name	Parent mass (Q1) $[M+H]^+$	Product mass (Q3)	Time (msec.)	DP (V)	EP (V)	CE (V)	CXP (V)
S-ZPC	389.200	245.000	200	55	8	25	17
R-ZPC	389.200	245.000	200	55	8	25	17
S-ZPC d8	397.000	245.000	200	20	4	20	17

MRM, multiple reaction monitoring; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

Preparation of stock and standard solutions: Working solutions of Calibration standards (CC) and quality control samples (QC) were prepared

from two separate primary stock solutions of both enantiomers (S-ZPC and R-ZPC).

Analyte stock solutions of S-Zopiclone, R-Zopiclone and internal standard S-Zopiclone-d8 were prepared in methanol with concentrations of 200.000 µg/mL. CC and QC stock solutions were diluted with methanol: milli-Q-water (50:50) to produce working solutions of 0.150, 0.350, 0.900, 1.300, 1.600, 2.000 (µg/mL) for CC and 0.010, 0.030, 0.850, 1.500, 3.000 (µg/mL) for QC samples. Working solutions were used to prepare plasma calibration standards and Quality control samples. Calibration standards in plasma were prepared by freshly spiking 190µL of control human plasma with the appropriate working solution of the analyte (10µL) and vortexed for complete mixing. Both enantiomers of Analyte (S-ZPC and R-ZPC) and internal standard (S-Zopiclone-d8) stock and working solutions were stored at 2-10°C. Samples for the determination of stabilities and effects were prepared by spiking control human plasma in bulk with S-ZPC and R-ZPC at appropriate concentrations [0.500 (LLOQ), 1.500 (LQC), 42.500 (MQC) and 75.000 (HQC) ng/mL)] and 300 µL aliquots were distributed in different tubes. The stability samples were stored at -70 °C and -20°C.

Sample preparation: For fresh spiking, (for preparing calibration standards and quality control samples) to 190 µL of plasma, 10µL of working solution (WCS -mixture of S-ZPC and R-ZPC) was added followed by vortexing. For bulk spiking (stability samples) and for subject samples, 200µL of sample was aliquoted. 10µL of WIS solution was added to all the samples and vortexed for 30 seconds for complete mixing. 200µL of 0.5% Formic acid buffer solution was added and vortexed. Samples were centrifuged at 15,000RPM for 5 minutes at 10^{o}C. Samples were loaded on to the SPE cartridges (Orochem, 30mg, 1mL) pre-conditioned with 1mL Methanol followed by equilibration with 1mL Milli Q Water. Cartridges were washed with 1mL Milli Q Water twice, followed by elution with 1mL of Mobile phase. Samples were vortexed and transferred into HPLC vials for analysis.

Validation of the assay method: The validity of the assay method was assessed according to regulatory guidelines (US DHHS, FDA, CDER, 2001), with regards to the linearity, sensitivity, precision, accuracy, recovery and stability.

The QC samples at three concentrations (low, medium and high-LQC, MQC, HQC) were used for these tests. For construction of the standard calibration curve, working solutions (mixture of S-Zopiclone and R-Zopiclone) were spiked into blank plasma to obtain concentrations of 0.500, 1.000, 3.500, 7.500, 17.500, 45.000, 65.000, 80.000 and 100.000 ng/mL. The between-run precision and accuracy of the method was evaluated at concentrations of 0.500, 1.500, 42.500 and 75.000 ng/mL. The within-run precision and accuracy of the method was evaluated at concentrations of 0.500, 1.500, 42.500, 75.000, and 100.000 (ng/mL). To evaluate ruggedness of the method, experiments using different analyst and different column were performed.

Linearity and Sensitivity: Calibration standards were prepared and analyzed at each concentration. Calibration curve was constructed using the analyte/IS peak area ratio versus the analyte's nominal concentration and fitted by linear least-squares regression analysis with weighting factor $1/x^2$. Sensitivity of the method was evaluated in terms of the lower limit of quantification (LLOQ). LLOQ was determined based on two criteria: (1) the analyte response at the LLOQ should be at least 5 times the response compared to blank response and (2) analyte peak (response) should be identifiable, discrete, and reproducible with a precision within ≤20% and accuracy of 80–120%.

Precision and Accuracy: Six replicates of QC samples were analyzed in the same run to determine within-run precision and accuracy, while in 4 separate runs QC samples were analyzed to determine the between-run precision and accuracy. Precision was calculated as the Coefficient of Variation (% CV), whereas accuracy was assessed as the percentage to the nominal concentration (% Nominal). The within and between-run %CV were ≤15% for low, medium and high QC samples and ≤20% for LLOQ samples at LLOQ concentration and ≤15% for other QC samples from respective nominal concentrations.

Recovery: Extraction recoveries of S-Zopiclone, R-Zopiclone and IS were assessed by comparing the peak areas of the extracted QC samples to the

post-extracted sample solutions containing equivalent amount of the analyte and IS. Briefly, 200µL of 0.5% Formic acid buffer solution and 10µL of 50% Methanol in Milli-Q-Water (Diluent for working standard solutions) solution were added to 190µL of blank plasma and vortexed. Samples were centrifuged at 15,000 RPM for 5 minutes at 10^{o}C. Samples were loaded on to the cartridges (Orochem, 30mg, 1mL) which were preconditioned with 1mL Methanol followed by equilibration with 1mL milli Q water. Cartridges were washed with 1mL milli Q water twice, followed by elution with 1mL of mobile phase. Samples were vortexed and transferred into HPLC vials for analysis.

To 980µL of above extracted elution solution, 10µL of QC working solution and 10µL of IS working solution were added and vortexed (post-extracted QC samples). Samples were vortexed and transferred into HPLC vials for analysis. The peak area, representing 100% recovery, was compared with that of the extracted QC samples.

Matrix effect: To determine the matrix effect, the post-extracted samples of six blank matrices, including one sample each of Hemolysed and Lipemic at LQC level, and aqueous LQC samples (six injection of aqueous sample) were analysed for assessing the lot-to-lot matrix effect. Post-extracted samples were prepared at LQC level by spiking WLQC as well as WIS in the processed blank matrices. One aqueous LQC sample (pure solution of the Analyte) was prepared by spiking WLQC as well as WIS in the similar manner that as of post-extracted samples. The processed post-extracted samples and aqueous sample (six injection of aqueous sample) were analyzed in a single run. Similarly matrix factor (MF) of analyte and IS was calculated. The IS normalized MF was also calculated by dividing the MF of the analyte by the MF of the IS.

Stability: The solution stability (Long Term Stock and Working Solutions Stability) and auto-sampler stability of S-Zopiclone, R-Zopiclone and IS were evaluated. Stability was also evaluated for S-Zopiclone and R-Zopiclone in Plasma samples (under dry ice, long term in matrix, bench top at room temperature and freeze thaw stability). For freeze thaw stability assessment, QC samples were exposed to five freeze (-70°C) and thaw (at room temperature) cycles and then analyzed along with the freshly prepared QC samples. For auto-sampler stability, the prepared samples in the auto-sampler were evaluated by analyzing the samples after being placed in the auto-sampler at 10°C for approximately 52 hours. For bench top stability**,** the QC samples were prepared and kept at room temperature for approximately 04 hours and then analyzed along with the freshly prepared samples. For dry ice stability, QC samples were prepared and kept under dry ice for approximately 77 hours and then analyzed along with the freshly prepared samples. All these stability tests were performed at LQC and HQC concentrations, each with six replicate samples.

Long Term Stability of Analyte and Internal Standard in Stock and Working Solutions were evaluated by injecting samples, each from stability stock, comparison stock and working solutions. Long Term Stability of Analyte in matrix was determined by analyzing stability samples (six samples each of LQC and HQC stored at -20°C and -70°C freezer) along with the fresh calibration standards and six samples each of LQC and HQC (comparison samples).

The percent change in concentration, over time, was used as an indicator of stability. The analyte was considered to be stable when the percent change was within ±15% of their respective nominal concentrations, in case of stock and working solution stability, the analyte and internal standard were considered to be stable when the percent change was within ±10% of the nominal concentration.

Other experiments: Injector carryover and Dilution integrity tests were performed. For dilution integrity test, six replicates of the Diluted Quality Control (DQC, 1.5*ULOQ concentration) were diluted 1/5th and 1/2nd respectively with human EDTA plasma prior to extraction and analysis for evaluating dilution integrity. To evaluate the re-injection reproducibility, LQC and HQC samples of one P&A experiment were kept in auto-sampler after analysis at 10°C, and re-injected after 2 hours. Concentrations were calculated to determine % change after re-injection. Hemolysis and anticoagulant effects were performed to check the effect of hemolysis and different anticoagulant (other than EDTA) on precision and accuracy of

samples. One set of samples (six samples each of LQC and HQC) was prepared in hemolysed blank EDTA plasma and another set was prepared in plasma containing Na-heparin as an anticoagulant. All samples were processed and analyzed with freshly processed calibration standards in normal EDTA plasma in a single run.

Human Pharmacokinetic study: A pharmacokinetic study was performed in normal, healthy, adult human subjects (n=24) under fasting condition. An Independent Ethics Committee (Ethics R'US, DN Nagar, Andheri West, Mumbai 400053) approved the protocol and the volunteers provided written informed consent as per ICH-GCP and local regulations. All subjects were under overnight fasting of at least 10 hours prior to the dosing and 4 hours after dosing. The subjects were administered a single dose of zopiclone 7.5 mg tablet with 240 mL of water. This activity was followed by oral examination to assess compliance to dosing.

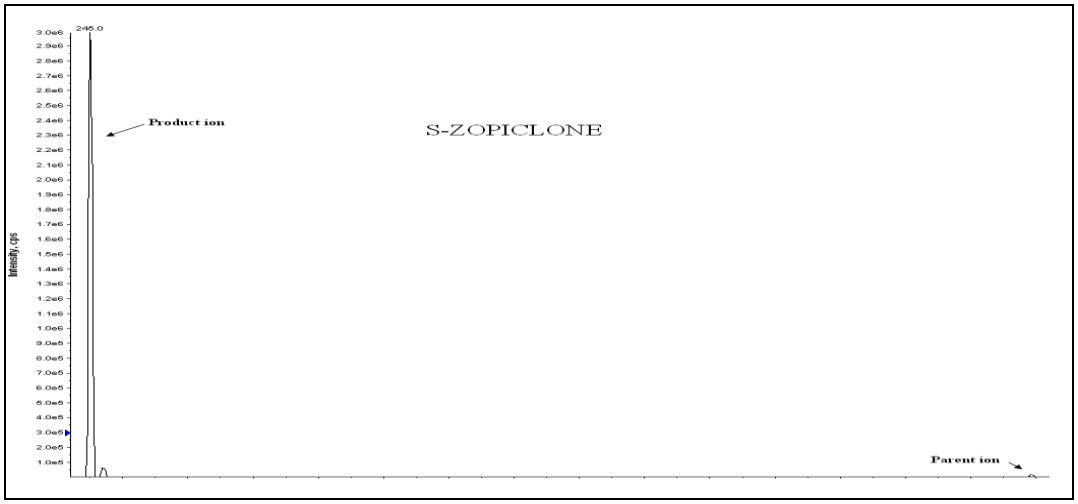
Subjects remained supine or semi-recumbent for at least 8 hours post-dose starting no longer than 15-30 minutes after dosing. Blood samples were collected in polypropylene tubes containing sodium EDTA solution as an anti-coagulant pre-dose and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 3.00, 4.00, 6.00, 9.00, 12.00, 15.00 and 24.00 hours after drug administration. The total volume collected per subject in this study did not exceed 251 ± 10 mL including 8 mL to 10 mL for screening, 5 mL to 7 mL for safety analysis and 0.5 mL of 'discarded' anti-coagulant mixed blood prior to each in-house sampling, i.e., total volume of discarded blood was 18 mL. Plasma was harvested by centrifuging the blood.

Harvested plasma samples were transferred from clinical pharmacology unit to the analytical unit under frozen conditions under dry ice. Analysis of study samples was performed as per GLP requirements. Plasma (200 µL) samples were spiked with IS and processed (as described in sample preparation) along with clinical samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among calibrators and unknown samples in the analytical run; not more than 33% of the QC samples were greater than ± 15% of the nominal concentration. Pharmacokinetic analysis was performed on plasma concentrations and time data of S-Zopiclone and R-Zopiclone using non-compartmental model of WinNonLin® Enterprise version 5.3 Pharsight USA.

RESULTS AND DISCUSSION:

Method development and validation: The goal of this work was to develop and validate a simple, sensitive, rapid and robust assay method for the quantitative determination of ZPC enantiomers from human plasma samples by using LC-MS/MS which is the most powerful analytical tool in clinical sample analysis for its selective, sensitive, reproducible and rapid analysis.

Optimization of Mass spectrometry: The full Q1 and Q3 scans of S-Zopiclone, R-Zopiclone and IS were acquired in positive ion mode by infusing the standard solutions of 100.000 ng/mL concentration prepared in Mobile phase and 50% methanol in water, respectively into ESI source. The Product ion mass spectra of these three compounds are shown in **Figure 2**.



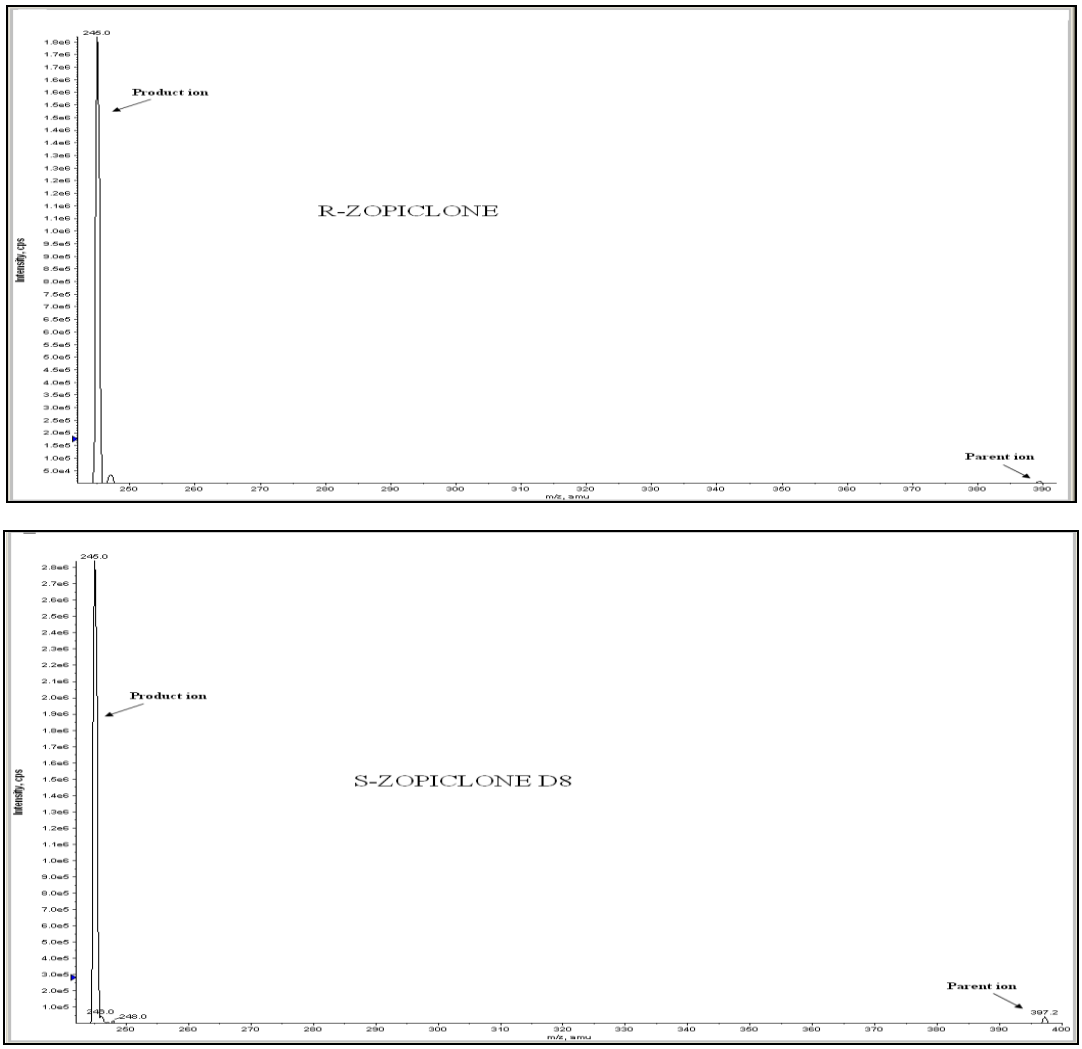


FIGURE 2: PRODUCT ION MASS SPECTRA OF S-ZOPICLONE, R-ZOPICLONE AND S-ZOPICLONE D8

Optimization of Chromatography: Initially, we tried with different extraction techniques like, LLE, Precipitation techniques.

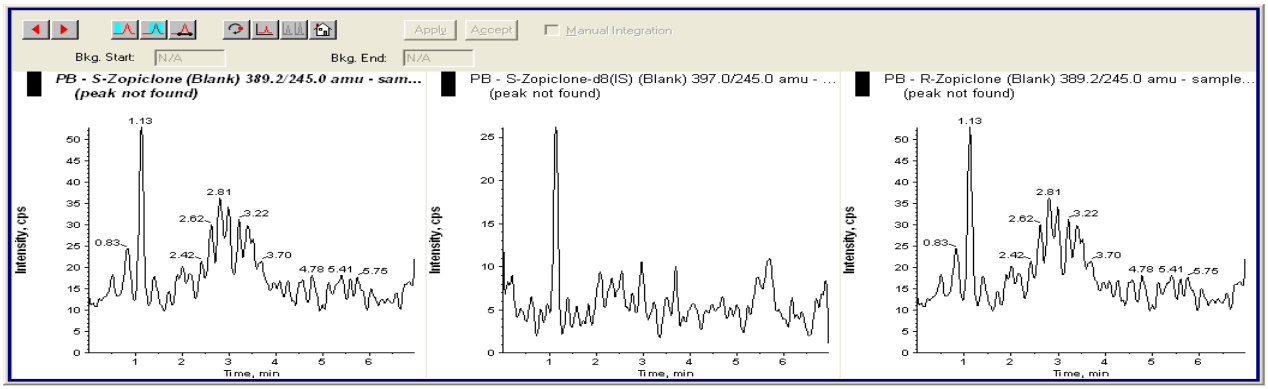
Finally, Solid Phase Extraction was selected as suitable extraction technique for drug and IS in terms of its greater recovery and reproducibility. Chromatographic conditions especially, the composition and nature of the mobile phase, different columns were optimized through several trials to achieve best resolution to increase the signal of S-ZPC, R-ZPC and S-Zopiclone d8. A good separation and elution was achieved with Chiralpak IC-3, 0.46 cm I.D*15 cm L column and 0.1% Ammonia in Acetonitrile and and 0.1% Ammonia in milli Q water (90:10 v/v) as the mobile phase at a flow-rate of 1.0 mL/min with 5 µL of injection volume.

Selectivity and specificity of validated method: Selectivity was performed by using 10 different lots of human plasma including Lipemic and Heamolyzed.

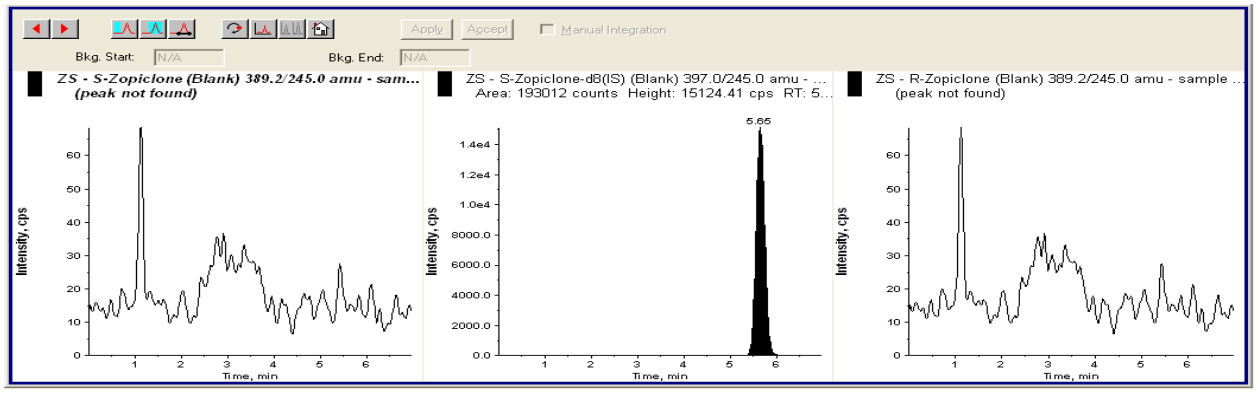
No significant interference was observed in either of the lots. Representative chromatograms of Plasma Blank, Zero Standard, Lower Limit of Quantitation, Upper Limit of Quantitation along with IS are displayed in **Figure 3**. The typical retention times of S-Zopiclone, R-Zopiclone and S-Zopiclone d8 were 5.68min, 4.04 min and 5.65 min respectively.

These result shows that S-Zopiclone and R-Zopiclone at high and low concentrations can be clearly detected. No significant interfering peaks at the retention time of S-Zopiclone, R-Zopiclone and S-Zopiclone d8 were observed, which shows that there was a lack of interference observed (from the endogenous components in plasma) at the corresponding peaks of S-Zopiclone, R-Zopiclone and S-Zopiclone d8.

No significant injector carry-over was observed at the RT of both Analytes and IS.



(3a)



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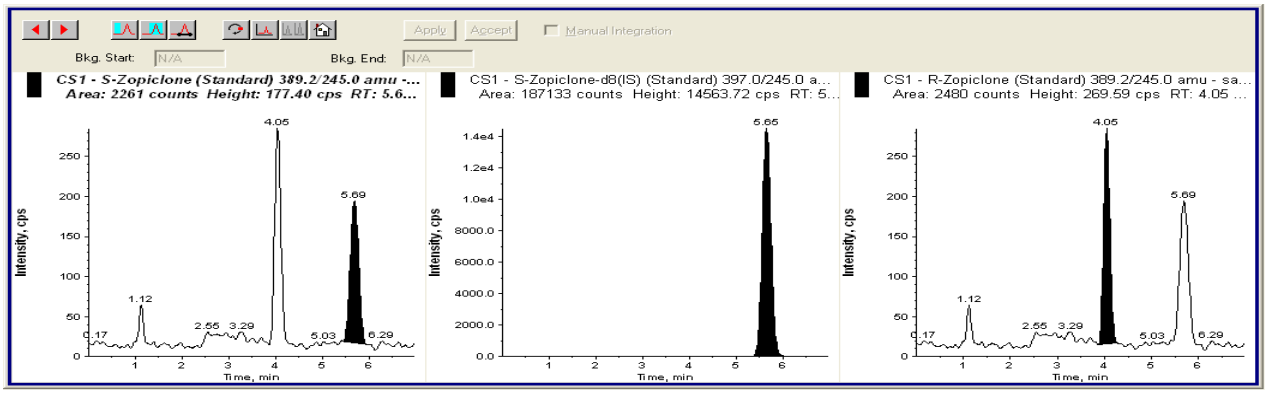
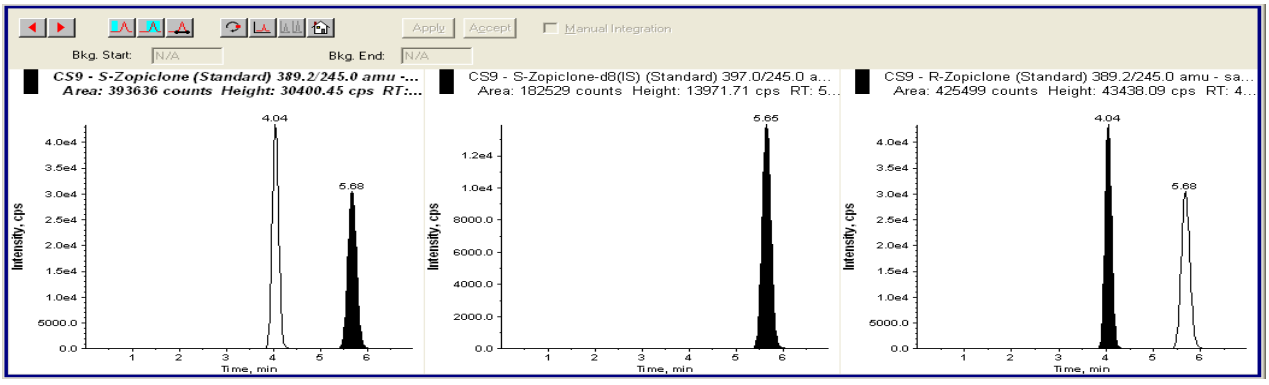


(3c)



(3d)

FIGURE 3: TYPICAL CHROMATOGRAMS OF S-ZOPICLONE (LEFT PANEL), IS (MIDDLE PANEL) AND R-ZOPICLONE (RIGHT PANEL) IN (3A) HUMAN BLANK PLASMA (3B) HUMAN PLASMA SPIKED WITH IS (3C) HUMAN PLASMA SPIKED WITH S-ZOPICLONE AND R-ZOPICLONE AT LLOQ LEVEL WITH IS, AND (3D) HUMAN PLASMA SPIKED WITH S-ZOPICLONE AND R-ZOPICLONE AT ULOQ LEVEL WITH IS

Linearity, Sensitivity, Precision and Accuracy: The calibration curves of S-Zopiclone and R-Zopiclone was linear over the concentration range of 0.500 ng/mL to 100.000 ng/mL and is shown in *Figure 4*. The correlation coefficient was greater than 0.9979 for multiple analytical runs. The best-fit line of the calibration curve was obtained by using a weighting factor of $1/x^2$.

The mean linear regression equations (n = 4) for S-Zopiclone and R-Zopiclone were: Y= 0.0219(±0.0005) X+ 0.0012((±0.0003), (r = 0.9995±0.0002)- and Y= 0.0224(±0.0015)X+ 0.0013((±0.0007), (r = 0.9990±0.0008) respectively. The % CV was less than 7% and accuracy of all CC standards ranged from 96.84 to 102.63 as shown in **Tables 2 & 3**.

For S-ZPC, R-ZPC QC samples, the between-run and within-run coefficients of variation is less than 9% and percentage of nominal value ranged from 84.19 to 102.99% as shown in **Tables 4 & 5**.

The method was found to be rugged with different analyst, and with different column of same make and specifications. These results show that the method is accurate, precise and reproducible for estimation of S-Zopiclone and R-Zopiclone in human plasma samples.

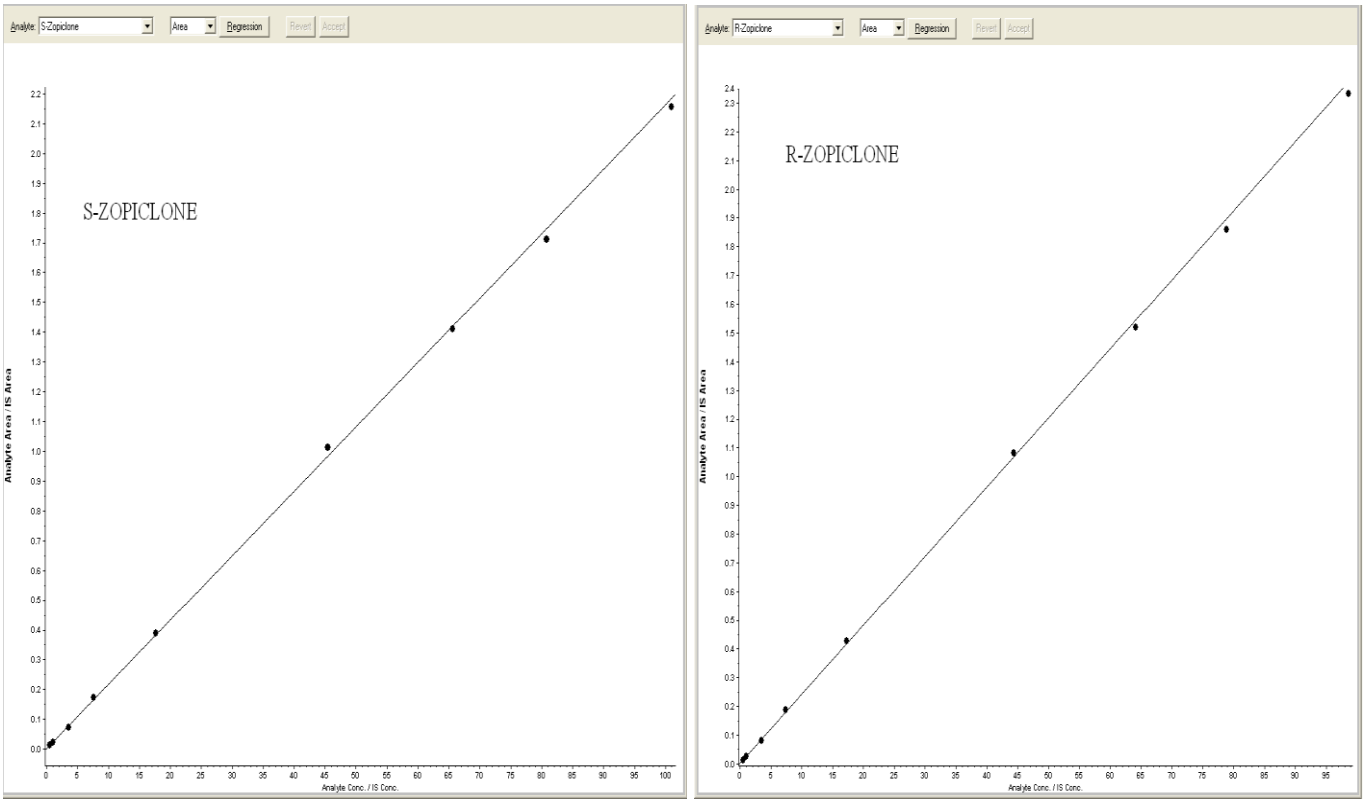


FIGURE 4: CALIBRATION CURVES OF S-ZOPICLONE AND R-ZOPICLONE IN HUMAN PLASMA

TABLE 2: CALIBRATION CURVE DETAILS FOR S-ZOPICLONE

CC ID	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9
Nominal Conc. (ng\mL)	0.505	1.01	3.535	7.575	17.676	45.452	65.653	80.804	101.005
Accepted Conc Range	(0.404-0.606)	(0.859-1.162)	(3.005-4.065)	(6.439-8.717)	(15.024-20.327)	(38.634-52.270)	(55.805-75.501)	(68.683-92.924)	(85.854-116.155)
N	4	4	4	4	4	4	4	4	4
Mean	0.504	1.015	3.501	7.738	17.541	46.021	64.797	80.936	100.197
S.D.	0.00	0.02	0.18	0.19	0.49	2.11	0.57	2.37	1.31
% C.V.	0.86	2.18	5.25	2.48	2.81	4.59	0.89	2.93	1.31
% Nominal	99.80	100.49	99.03	102.14	99.24	101.25	98.7	100.16	99.2

TABLE 3: CALIBRATION CURVE DETAILS FOR R-ZOPICLONE

CC ID	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9
Nominal Conc. (ng\mL)	0.493	0.986	3.452	7.397	17.26	44.383	64.109	78.903	98.629
Accepted Conc Range	(0.395-0.592)	(0.838-1.134)	(2.934-3.970)	(6.288-8.507)	(14.671-19.849)	(37.726-51.041)	(54.493-73.725)	(67.068-90.739)	(83.835-113.423)
N	4	4	4	4	4	4	4	4	4
Mean	0.485	1.012	3.489	7.563	17.247	44.652	62.084	78.205	97.774
S.D.	0.01	0.06	0.17	0.18	0.41	2.30	1.55	2.47	2.11
% C.V.	3.57	6.67	4.85	2.34	2.39	5.15	2.5	3.17	2.15
% Nominal	98.40	102.63	101.08	102.25	99.92	100.6	96.84	99.12	99.13

TABLE 4: PRECISION AND ACCURACY FOR S-ZOPICLONE (ANALYSIS WITH SPIKED PLASMA SAMPLES AT FIVE DIFFERENT CONCENTRATIONS FOR WITHIN-RUN AND FOUR DIFFERENT CONCENTRATIONS FOR BETWEEN-RUN)

Spiked plasma concentration (ng/mL)	Within-run			Between-run		
	Concentration measured (n=6) (ng/mL) (mean±S.D.)	%CV	% Accuracy	Concentration measured (n=24) (ng/mL) (mean±S.D.)	%CV	% Accuracy
0.505	0.487±0.03	5.68	96.33	0.518±0.05	8.83	102.64
1.509	1.516±0.07	4.39	100.42	1.541±0.07	4.68	102.09
42.758	44.036±0.93	2.11	102.99	44.017±1.25	2.83	102.94
75.455	75.659±1.40	1.85	100.27	76.432±2.41	3.15	101.30
101.005	98.769±2.19	2.22	97.79	N/A		

TABLE 5: PRECISION AND ACCURACY FOR R-ZOPICLONE (ANALYSIS WITH SPIKING PLASMA SAMPLES AT FIVE DIFFERENT CONCENTRATIONS FOR WITHIN-RUN AND FOUR DIFFERENT CONCENTRATIONS FOR BETWEEN-RUN)

Spiked plasma concentration (ng/mL)	Within-run			Between-run		
	Concentration measured (n=6) (ng/mL) (mean±S.D.)	%CV	% Accuracy	Concentration measured (n=24) (ng/mL) (mean±S.D.)	%CV	% Accuracy
0.493	0.415±0.02	5.20	84.19	0.463±0.04	8.43	93.870
1.488	1.426±0.09	6.12	95.82	1.436±0.08	5.55	96.473
42.164	42.269±0.76	1.83	100.25	41.697±1.46	3.50	98.892
74.407	72.868±1.43	1.97	97.93	72.632±3.09	4.26	97.613
98.629	94.100±2.30	2.45	95.41	N/A		

Recovery: The recovery of S-ZPC, R-ZPC was determined at three different concentrations levels and obtained mean recoveries were found to be 87.30%, 90.14%, 88.87% and 81.67%, 85.80%, 85.37% at low, medium and high quality control levels respectively. The average recovery of S-ZPC, R-ZPC and S-Zopiclone-d8 were found to be 88.77%, 84.28% and 89.12% respectively.

Matrix Effect: The recovery yield of this method shows that recovery rate was consistent over the calibration range.

No effect of matrix (six different lots of EDTA plasma including one sample each of Hemolysed and Lipemic) was observed on analyte quantitation.

The overall precision of the matrix factor is expressed in terms of Coefficient of Variation (% CV) and was determined to be 4.22 for S-ZPC, 4.02 for R-ZPC and 1.09 for S-Zopiclone-d8. And Coefficient of Variation of Internal Standard Normalized Matrix Factor for S-Zopiclone and R-Zopiclone were 4.76% and 4.73%, respectively.

Stability (Bench Top, Freeze-Thaw, Coolant, Auto-Sampler Stabilities): Both the enantiomers were found to be stable in plasma under the storage conditions (-70°C and -20°C) for at least 81 days, and room temperature for approximately 04 hours which is long enough to cover the whole study duration. Upon five freeze thaw cycles, almost no difference was observed in the peak areas of QC samples in comparison to the freshly prepared samples. No significant degradation of S-Zopiclone, R-Zopiclone and S-Zopiclone d8 was observed when the extracted samples were kept in the auto-sampler at 10°C for approximately 52 hours. S-Zopiclone and R-Zopiclone were found stable under dry ice for approximately 77 hours. For S-Zopiclone, R-Zopiclone and S-Zopiclone d8, stock and working solutions were found to be stable for 45 days. The obtained concentrations for S-Zopiclone and R-Zopiclone at 1.509,75.455 ng/mL and 1.488,74.407 ng/mL were within ±15% of the respective nominal concentrations in a series of stability tests details were mentioned in Table 6 & 7, and long term freezer stability at temperatures of -70 & -20°C for at least 81 days . The results were found to be within the assay variability limits during the entire process.

TABLE 6: STABLITY OF S-ZOPICLONE IN HUMAN PLASMA

Stability Name	Bench Top (Room Temperature) Stability		Freeze-Thaw Stability at -70^{0}C		Coolant Stability (Under dry ice)		Auto-sampler Stability at 10^{0}C	
Duration Time	04 hours 35 mins		5 Cycles		77 hours 55 mins		52 hours 18 mins	
Spiked plasma conc. (ng/mL)	**Conc. measured (n=6) (ng/mL) (mean±S.D.)**	**% CV**	**Conc. measured (n=24) (ng/mL) (mean±S.D.)**	**% CV**	**Conc. measured (n=6) (ng/mL) (mean±S.D.)**	**% CV**	**Conc. measured (n=24) (ng/mL) (mean±S.D.)**	**% CV**
1.509	1.561±0.04	2.59	1.537±0.07	4.47	1.604±0.09	5.49	1.560±0.03	1.63
75.455	77.473±1.73	2.23	76.853±2.11	2.74	76.913±1.38	1.8	77.383±1.82	2.35

TABLE 7: STABLITY OF R-ZOPICLONE IN HUMAN PLASMA

Stability Name	Bench Top (Room Temperature) Stability		Freeze-Thaw Stability		Coolant Stability		Auto-sampler Stability	
Duration Time	04 hours 35 mins		5 Cycles		77 hours 55 mins		52 hours 18 mins	
Spiked plasma conc. (ng/mL)	**Conc. measured (n=6) (ng/mL) (mean±S.D.)**	**% CV**	**Conc. measured (n=24) (ng/mL) (mean±S.D.)**	**% CV**	**Conc. measured (n=6) (ng/mL) (mean±S.D.)**	**% CV**	**Con measured (n=24) (ng/mL) (mean±S.D.)**	**% CV**
1.488	1.421±0.06	4.56	1.415±0.05	3.74	1.438±0.02	1.32	1.436±0.05	3.15
74.407	73.760±1.78	2.41	73.912±2.26	3.06	70.831±2.02	2.85	70.497±2.28	3.23

Other experiments: No significant Injector carryover was observed. The results have shown that the precision and accuracy of diluted samples were within acceptable range. S-Zopiclone and R-Zopiclone were found to be stable in auto-sampler at 10°C and reproducible after re-injection. No effect of hemolysis and anticoagulant was observed on analyte quantitation. Formulae used for calculation are given in **Table 8**. Results of method validation are summarized in **Table 9**.

TABLE 8: FORMULAE USED IN VALIDATION

Mean:	$\frac{\text{Sum of All Values}}{\text{Number of Values}}$
Standard Deviation [SD]:	$\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$ .... $divisor\ n-1$

Precision: Coefficient of Variation [C.V. (%)]:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

Accuracy: % Nominal Concentration:

$$\frac{\text{Concentration Found}}{\text{Nominal Concentration}} \times 100$$

% of Recovery:

$$\frac{\text{Mean Peak Response of Extracted Samples}}{\text{Mean Peak Response of Unextracted Samples}} \times 100$$

% of Change:

$$\left\{\frac{\text{Mean or Corrected Mean Conc of Stability Samples}}{\text{Mean or Corrected Mean Conc of Comparison Samples}}\right\} - 1 \times 100$$

Ratio Corrected for Concentrations:

$$\frac{\text{Peak Response Ratio of Stability Samples X Comparison Sample Concentration}}{\text{Stability Sample Concentration}}$$

% of Change of Stock Solutions:

$$\left\{\frac{\text{Ratio Corrected for Concentration (Stability Sample)}}{\text{Mean Peak Response Ratio (Comparison Samples)}}\right\} - 1 \times 100$$

TABLE 9: SUMMARY OF THE VALIDATED METHOD

EXPERIMENT	PARAMETER	S-ZOPICLONE	R-ZOPICLONE
Dilution Integrity accuracy	QC % Nominal value	98.18 to 98.84	96.47 and 96.78
Dilution Integrity precision	QC Coefficient of variation	2.64 to 2.82%	3.00 to 3.22%
Hemolysis effect accuracy	QC % Nominal value	101.34 to 104.70	98.32 to 99.82
Hemolysis effect precision	QC Coefficient of variation	1.98 to 5.22%	2.47 to 2.54%
Anticoagulant effect accuracy	QC % Nominal value	102.65 to 104.01	99.33 to 100.61
Anticoagulant effect precision	QC Coefficient of variation	1.94 to 4.88%	1.67 to 3.01%
Short term stability of analyte in stock solution on bench	Mean % Change after 16 Hours 35 minutes	0.34	1.00
Short term stability of internal standard in stock solution on bench	Mean % Change after 16 Hours 51 minutes	2.38	2.38
Short term stability of analyte in working solution on bench	Mean % Change after 16 Hours 40 minutes	-1.71	-1.86
Short term stability of internal standard in working solution on bench	Mean % Change after 16 Hours39 minutes	0.81	0.81
Long Term stability of analyte in stock solution at 2-10°C	Mean % Change after 46 days	1.15	2.96
Long Term stability of internal standard in stock solution at 2-10°C	Mean % change after 46 days	1.10	1.10
Long Term stability of analyte in working solution at 2-10°C	Mean % change after 45 days	3.76	4.37
Long Term stability of internal standard in working solution at 2-10°C	Mean % Change after 45 days	2.58	2.58
Long Term stability of Analyte in matrix at -20±10ºC	QC Mean % change after 81 days	-0.96 to 5.31%	-4.90 to 4.14%
Long Term stability of Analyte in matrix at -70±15ºC	QC Mean % change after 81 days	-0.73 to 0.45%	-1.58 to 0.61%
Ruggedness- Different analyst: accuracy	QC % Nominal value	103.10 to 105.24	98.48 to 100.03
Ruggedness- Different analyst: precision	QC Coefficient of variation	1.37 to 8.39%	2.67 to 8.10

Human pharmacokinetic study: The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of S-ZPC and R-ZPC (following administration of Zopiclone 7.5 mg tablets) in healthy volunteers. Profiles of the mean plasma concentration versus time of S-ZPC and R-ZPC were shown in *Figure 5*. S-ZPC attained peak plasma concentrations (46.58 ± 9.62 ng/mL) at 1.04 ± 0.92 h, whereas R-ZPC maximum plasma concentrations (33.97 ± 7.38 ng/mL) achieved at 1.00 ± 0.89 h. The AUC_{0-t} values for S-ZPC and R-ZPC were found to be 285.91 ± 42.78 and 128.44 ± 20.87 ng.h/mL, respectively. The terminal half-life ($t_{½,\beta}$) was found to be 6.68 ± 1.33 and 3.89 ± 0.79 h for S-ZPC and R-ZPC, respectively. The higher sensitivity of this method enabled the quantitation of S-ZPC and R-ZPC to calculate the PK parameters. The obtained PK parameters for S-ZPC and R-ZPC in human subjects matched with published data [29]. Hence we came up with this new validated, sensitive, simple, high throughput LC-MS/MS method in order to establish the PK parameters for S-ZPC and R-ZPC in human subjects following administration of Zopiclone 7.5 mg tablet in human subjects.

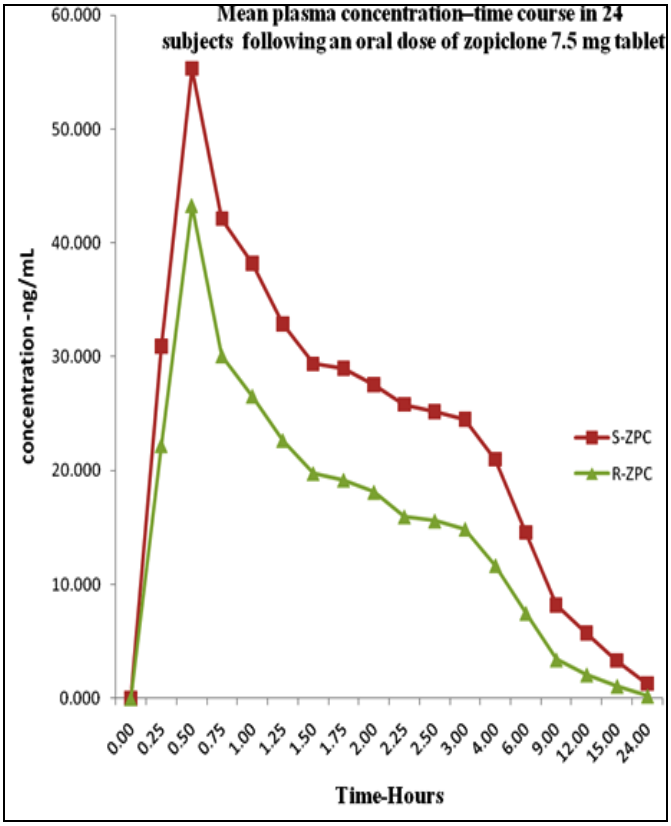


FIGURE 5: MEAN PLASMA CONCENTRATION–TIME COURSE IN 24 SUBJECTS FOLLOWING AN ORAL DOSE OF ZOPICLONE 7.5 MG TABLET

CONCLUSION: A highly sensitive analytical method was developed and validated for the first time in human plasma for the simultaneous quantification of Zopiclone enantiomers and was successfully applied to perform sample analysis of a pharmacokinetic study by using LC-MS/MS. The results indicate that this method is suitable for pharmacokinetic and bioavailability studies.

This method offers various advantages over other published methods, such as less sample volume, high throughput, greater sensitivity and simplicity.

The present LC–MS/MS method provides a simple, robust, quick and sensitive analytical tool for simultaneous quantitation of Zopiclone enantiomers in human plasma and has been successfully applied to clinical pharmacokinetic study in human subjects.

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REFERENCES:

1. British Pharmacopeia 2013; volume II, Page No-2384
2. www.who.int/medicines/areas/quality_safety/4.6Zopiclone CritReview.pdf- Assessment of Zopiclone by WHO
3. Lanka A. Rama Prasad, J.V.L.N.S. Rao, Srinivasu Pamidi, Vara Prasad .J, Kishore Kumar Hotha- UPLC method for the determination of Eszopiclone and its related impurities. International Journal of Analytical and Bioanalytical Chemistry 2012; 2(4): 241-246
4. Fernandez C, Martin C, Gimenez F, Farinotti R. Clinical pharmacokinetics of zopiclone. Clinical Pharmacokinetics .1995; 29: 431-441.
5. Leonard B. An introduction to enantiomers in psychopharmacology. Hum Psychopharmacology 2001;16: S79-S84.
6. products.sanofi.ca/en/imovane.pdf - Submission Control No.: 146277-Imovane (zopiclone) - Sanofi Canada -Oct 13, 2011
7. Ohayon MM, Lader MH .Use of psychotropic medication in the general population of France, Germany, Italy, and the United Kingdom. The Journal of Clinical Psychiatry 2002; 63: 817-825.
8. Wagner J, Wagner ML. Non-benzodiazepines for the treatment of insomnia. Sleep Medicine Reviews 2000; Volume 4.
9. MG Gebauer, CP Alderman. Validation of a high performance liquid chromatographic method for the enantiospecific quantitation of zopiclone in plasma. Biomedical Chromatography 2002; 16: 241–246.

10. J Van Bocxlaer, E Meyer, K Clauwaert , W Lambert ,M Piette, A DeLeenheer, Analysis of zopiclone (Imovane) in postmortem specimens by GC–MS and HPLC with diode-array detection, Journal of Analytical Toxicology.1996;20: 52-54.
11. L Oiestad, U Johansen, AS Christophersen Drug screening of preserved oral fluid by liquid Chromatography–tandem mass spectrometry Clinical Chemistry 2007; 53: 300–309.
12. O Ouintela , FL Sauvage, F Charvier, JM Gaulier, G Lachatre , P Marquet, Liquid Chromatography–tandem mass spectrometry from detection of low concentration of 21 benzodiazepines, metabolites, and analogs in urine: method with forensic application Clinical Chemistry. 2006; 52: 1346–1355.
13. M Laloup M, M. Ramirez Fernanadez , G De Boeck, M Wood, N Maes,V . Samyn, Validation of a liquid chromatography–tandem mass spectrometry method for the simultaneous determination of 26 benzodiazepines and metabolites, zolpidem and zopiclone, in blood, urine and hair. Journal of Analytical Toxicology 2005; 29:616–626.
14. C Kratzsch , O Tenberken , FT Peters , AA Weber , T Kraemer , HH Maurer , Screening, library-assisted identification and validated quantification of 23 benzodiazepines, flumazenil, zaleplone, zolpidem and zopiclone in plasma by liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization. Journal of Mass Spectrometry 2004; 39: 856–872.
15. H Mistri, A Jangid, A Pudage and P Shrivastav HPLC-ESI-MS/MS validated method for simultaneous quantification of zopiclone and its metabolites, Ndesmethyl zopiclone and zopiclone-N-oxide in human plasma. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences.2008; 864: 137-48.
16. M Meng, L Rohde, V Cápka , SJ Carter , PK Bennett. Fast chiral chromatographic method development and validation for the quantitation of eszopiclone in human plasma using LC/MS-MS. Journal of Pharmaceutical and Biomedical Analysis 2010; 53(4):973-82
17. Hotha KK, Vijaya Bharathi D, Jagadeesh B, Ravindranath LK, Jaya Veera KN and Venkateswarulu V. A rapid LC-MS/MS method for quantitation of eszopiclone in human plasma: application to a human pharmacokinetic study. Biomedical Chromatography. 2011; 26: 225-231.
18. Dhaneshwar S and Bhusari V. Development of a Validated Stability-Indicating HPLC assay method for Eszopiclone. International Journal of ChemTech Research.2011; 3: 680-89.
19. K Anandakuma, G Kumaraswamy, T Ayyappan, A Sankar and D Nagavalli. Development of a Validated Stability-Indicating HPLC assay method for Eszopiclone. Asian Journal of Research in Chemistry. 2010; 3: 63-67.
20. RT Foster , G Caille AH Ngoc , CH Lemko , R Kherani , FM Pasutto Stereospecific high-performance liquid chromatographic assay of zopiclone in human plasma. Journal of Chromatography B 1994; 658:161–166.
21. Fernandez C, Alet P, Davrinche C, Adrien J, Thuillier A, Farinotti R, Gimenez F- Stereoselective distribution and stereoconversion of zopiclone enantiomers in plasma and brain tissues in rats. Journal of Pharmacy and Pharmacology 2002; Mar; 54(3):335-40.
22. Nilsson GH, Kugelberg FC, Kronstrand R, Ahlner J. Stability tests of zopiclone in whole blood. Forensic Sci Int. 2010 Jul 15;200(1-3):130-5
23. Ricarda Jantos, Annemiek Vermeeren, Danica Sabljic, Johannes G. Ramaekers, Gisela Skopp Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots International Journal of Legal Medicine January 2013; Volume 127(1): 69-76
24. Jones AW, Holmgren A. Concentrations of zolpidem and zopiclone in venous blood samples from impaired drivers compared with femoral blood from forensic autopsies. Forensic Sci Int. 2012 Oct 10; 222(1-3):118-23. doi:10.1016/j.forsciint.2012.05.008. Epub 2012 Jun 4.
25. RP-HPLC Method for the Estimation of Zopiclone in Tablet Dosage Form‖; International Journal of Pharmaceutical Research; 2011; 3(1): 49-51.
26. Sangaraju S, Lakshmi Kanth M, Rao BM, Someswararao N. Enantiomeric separation of S-zopiclone and its R-enantiomer in bulk drug samples by validated chiral RP-HPLC. Pharmazie.2009 Nov; 64(11): 717-9
27. Milena Araújo Tonon & Valquíria A. P. Jabor & Pierina Sueli Bonato- Enantioselective analysis of zopiclone and its metabolites in plasma by liquid chromatography/tandem mass spectrometry- Analytical and Bioanalytical Chemistry 2011; 400:3517–3525
28. ww.accessdata.fda.gov/drugsatfda.../021476_Lunesta_biopharmr.PDF.
29. www.drugbank.ca/drugs/DB01198 - Jun 13, 2005.

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