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Article

A Rapid and Highly Sensitive UPLC–MS-MS Method for the Quantification of Zolpidem Tartrate in Human EDTA Plasma and its Application to Pharmacokinetic Study

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A rapid and high sensitive liquid chromatography–tandem mass spectrometry (LC–MS-MS) method was developed and validated for the quantification of zolpidem in human EDTA plasma using ondansetron (IS) as an internal standard. The analyte and IS were extracted from human plasma using ethyl acetate and separated on a C18 column (Inertsil-ODS, 5 μm, 4.6 × 50 mm) interfaced with a triple quadrupole tandem mass spectrometer. The mobile phase, which consisted of a mixture of methanol and 20 mM ammonium formate (pH 5.00 ± 0.05; 75:25 v/v), was injected at a flow rate of 0.40 mL/min. The retention times of zolpidem and IS were approximately 1.76 and 1.22. The LC run time was 3 min. The electrospray ionization source was operated in positive ion mode. Multiple reaction monitoring used the $[M + H]^+$ ions m/z 308.13 → 235.21 for zolpidem and m/z 294.02 → 170.09 for the ondansetron, respectively. Five freeze-thaw cycles was established at −20 and −70°C.The linearity of the response/concentration curve was established in human EDTA plasma over the concentration range 0.10–149.83 ng/mL. The lower detection limit [(signal-to-noise (S/N) > 3] was 0.04 ng/mL and the lower limit of quantification (S/N > 10) was 0.10 ng/mL. This LC–MS-MS method was validated with intra-batch and inter-batch precision of 0.52–8.66.The intra-batch and inter-batch accuracy was 96.66–106.11. Recovery of zolpidem in human plasma was 87.00% and IS recovery was 81.60%. The primary pharmacokinetic parameters were T_{max} (h) = (1.25 ± 0.725), C_{max} (ng/mL) (127.80 ± 34.081), $AUC_{0\rightarrow t}$, = (665.37 ± 320.982) and $AUC_{0\rightarrow\infty}$, 686.03 ± 342.952, respectively.

Introduction

Zolpidem (Ambien) is a prescription medication used for the short-term treatment of insomnia (difficulty falling asleep or staying asleep) and some brain disorders. It is a short-acting non-benzodiazepine hypnotic that potentiates gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter, by binding to GABA receptors at the same location as benzodiazepines (1). It works quickly (usually within 15 minutes) and has a short half-life (2–3 hours). Zolpidem has not adequately demonstrated effectiveness in maintaining sleep; however, it is effective in initiating sleep (2). Its hypnotic effects are similar to those of the benzodiazepine class of drugs, but it is molecularly distinct from the classical benzodiazepine molecule and is classified as an imidazopyridine. Flumazenil, a benzodiazepine receptor antagonist, which is used for benzodiazepine overdose, can also reverse zolpidem's sedative/hypnotic and memory impairing effects (3, 4). Due to its selective binding, zolpidem has very weak anxiolytic, muscle relaxant, and anticonvulsant properties, but very strong hypnotic properties (5). Zolpidem tartrate, chemically *bis*[*N, N* dimethyl-2[6-methyl-2-(4-methyl phenyl)]imidazo[1, 2-α]pyridine-3-yl]acetamide](2R,3R)-2,3dihydroxybutane dioate, is a hypnotic agent (6) and a molecular weight of 764.88.

Several analytical methods have been reported for the quantification of zolpidem in human plasma using high-performance liquid chromatography (HPLC) (7), HPLC with fluorescence detection (8–10) and liquid chromatography with tandem mass spectrometry (LC–MS-MS) (11–12). The following are the advantages of the current method over those reported previously: (i) greater sensitivity is achieved (0.10 ng/mL), even with low plasma volumes, and the method is well suited for pharmacokinetic analysis; (ii) employing a single-step liquid–liquid extraction procedure minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure; (iii) because it uses less plasma volume (0.300 mL), the volume of the sample to be collected for the time point from subjects during the study is reduced significantly—this allows inclusion of additional points; (iv) the rapid sample analysis turnaround time of 3.00 min makes it an attractive procedure for high-throughput bioanalysis of zolpidem in human plasma. The chromatographic conditions were optimized and the validation results were provided in terms of specificity, linearity, precision, accuracy, extraction efficiency, dilution integrity and stabilities. The devised method was used in a zolpidem tartrate bioequivalence study, which was conducted in accord with United States Food and Drug Administration (USFDA) guidelines (13). Typical bioavailability parameters were compared, including the area under plasma concentration-time curve ($AUC_{0\rightarrow t}$), maximum plasma concentration (C_{max}) and area under the concentration time-curves from time zero to infinity, ($AUC_{0\rightarrow\infty}$).

Chemicals and reagents

Zolpidem tartrate (Figure 1) and ondansetron HCl (internal standard; IS) (Figure 2) was obtained from Aurobindo Pharma (Hyderabad, India). Methanol and HPLC-grade acetonitrile were purchased from J.T. Baker (Philipsburg, NJ). Analytical-grade ammonium formate was purchased from SD Fine Chemicals (Mumbai, India). Ethyl acetate and formic acid (AR grade) were

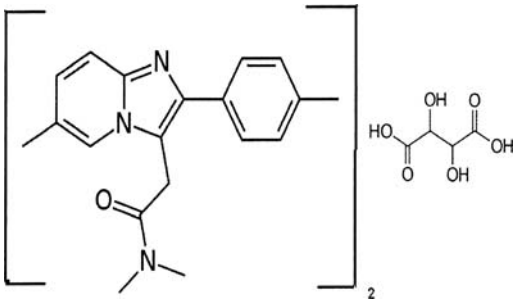

Figure 1. Chemical structure of zolpidem tartrate.

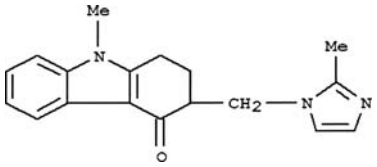

Figure 2. Chemical structure of ondansetron HCl: molecular formula, $C_{18}H_{19}N_3O \bullet HCl \bullet 2H_2O$; molecular weight, 365.86.

Table I
Optimized Mass Spectrometry Parameters for Analytes and IS

Channel	Parent (Da)	Daughter (Da)	Dwell (s)	Cone (V)	Collision (eV)
Zolpidem	308.13	235.21	0.5	50	35
Ondansetron HCl	294.02	170.09	0.5	35	31
ES^+ source					
Capillary (kv)					3.50
Cone (v)					50.00
Extractor (v)					2.00
RF lens (v)					0.0
Source temperature (°C)					100
Desolation temperature (°C)					400
Desolation gas flow (L/h)					50
Cone gas flow (L/h)					600
Analyzer					
LM resolution 1					14.5
HM resolution 1					14.5
Ion energy 1					0.5
Entrance					−1
Collision					35
Exit					1
LM resolution 2					14.0
HM resolution 2					14.0
Ion energy 2					1.0
Multiplier					650

purchased from RFCL Chemicals (New Delhi, India). The control human EDTA plasma sample was procured from Cauvery Diagnostics and Blood Bank (Secunderabad, India.) Polypropylene vials (Torsens Products, Kolkata, India). Milli-Q water used for the LC–MS-MS analysis was prepared using a Milli-Q water purification system procured from Millipore (Bangalore, India).

Instrumentation

A Waters ACQUITY UPLC System (Milford, MA) consists of a binary solvent manager, sample manager (including the column heater), detector and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. The system includes built-in solvent degassing and solvent select valves to choose from up to four solvents, as well as a 15,000 psi pressure limit (approximately 1,000 bar) to take full advantage of the sub-2-m particles. The sample manager also incorporates several technology advancements that were used in the study. Ionization and detection of the analyte and IS was carried out on a triple quadrupole mass spectrometer (Waters, Quattro Micro) equipped with electrospray ionization (ESI) and operating in the positive ion mode. Quantization was performed using the multiple reaction monitoring (MRM) mode to monitor parent → product ion (m/z) transitions: 308.13 → 235.21 for zolpidem and 294.02 → 170.09 for IS. The source-dependent parameters for zolpidem and ondansetron were: capillary, 3.50 kV; extractor, 2.00 V; RF lens, 0.0V; source temperature, 100°C; desolvation temperature, 400°C; cone gas flow, 50 ± 10 L/h; desolvation gas flow, 600 ± 10 L/h. The optimum values for compound-dependent parameters (MRM file parameters) such as cone voltage and collision energy were 50 V and 35 eV for the analyte and 35 V and 35 eV for IS, respectively. The dwell time was set at 500 ms. Mass Lynx software version 4.1 was used to control all parameters of UPLC and MS. Table I shows the mass parameters for both zolpidem and ondansetron. Figure 3A shows the product ion mass spectra of $[M + H]^+$ of zolpidem.

Chromatographic conditions

The separation of zolpidem and ondansetron was performed on an Inertsil-ODS, 3 V (4.6 × 50 mm, 5 μm) column and was maintained at 35°C in the column oven. The mobile phase consisted of 20 mM ammonium formate (pH 5.00 ± 0.05) and methanol in a 25:75 (v/v) ratio. For isocratic elution, the flow rate of the mobile phase was kept at 0.40 mL/min. The total chromatographic run time was 3.00 min. Sample temperature was maintained at 10°C and the pressure of the system was 800 psi.

Method development

Method development started by tuning the molecules. MS parameters were tuned in both positive and negative ionization modes for zolpidem and IS. However, the response was found to be good in positive ionization mode and quantification was achieved by MS-MS detection for the analyte and IS. A Waters Acquity UPLC interfaced with Waters Quattro Micro MS-MS was used as the chromatographic separation module. Separation was attempted using various combinations of acetonitrile, methanol and buffers with various contents of each component on a variety of columns like C8 and C18 of different makes such as Chromolith, Hypersil, X-terra, Kromasil, Intertsil and Grace. It was found that an isocratic mobile phase system consisting of 20 mM ammonium formate (pH 5.00 ± 0.05) with methanol (25:75, v/v) could achieve high responses and good peak shapes; therefore, it was finally adopted as the mobile phase that was degassed ultrasonically for 10 min. A flow rate of 0.400 mL/min (without splitter) into the ESI-MS chamber produced good peak shapes and permitted a run time

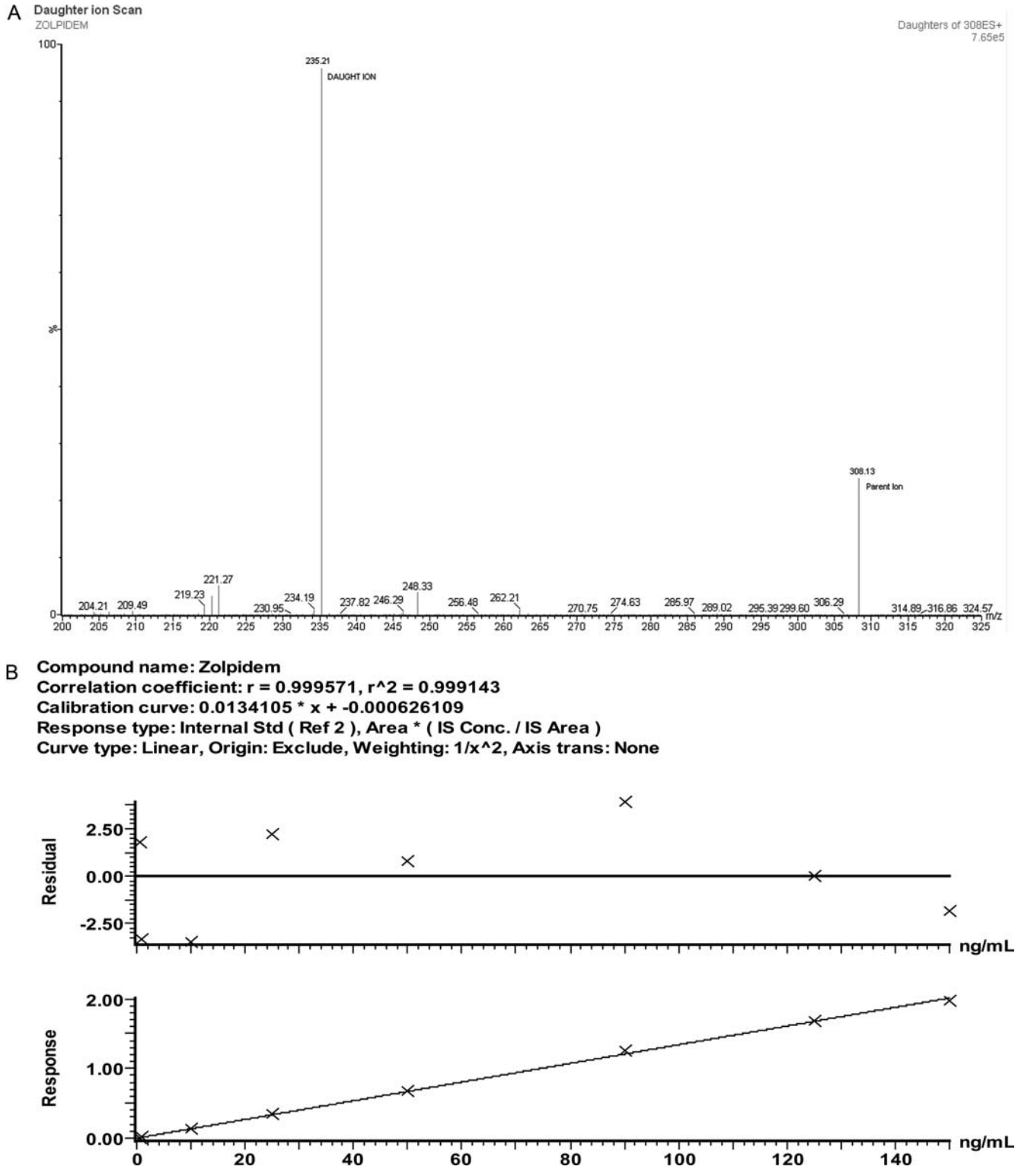

Figure 3. Product ion mass spectra of $[M + H]^+$ of zolpidem (A); calibration curve of zolpidem (B).

of only 3.0 min. A 10-μL injection volume of the processed sample was injected onto the Inertsil-ODS column (4.6 × 50 mm, 5 μ; GL Sciences, Japan), at a column oven temperature of 35 ± 2°C and autosampler temperature of 10 ± 2°C. The retention times of zolpidem and IS were 1.76 ± 0.35 and 1.22 ± 0.24 min. Consistent extraction efficiency using direct precipitation and liquid–liquid extraction techniques was sought for the analyte and selected IS. The direct precipitation

technique (DPT) was surpassed by liquid–liquid extraction to avail a desired volume of reconstitution and to eliminate matrix effect. Clean samples are essential for minimizing ion suppression and matrix effect in LC–MS-MS. Several solvents were checked alone and in combination, although ethyl acetate provided the best results in terms of recovery, matrix effect and process efficiency. The Inertsil-ODS (4.6 × 50 mm) column was selected because it could provide a clean chromatogram for a blank sample that yielded a good, reproducible response with better matrix effect results. The selected mobile phase proved to be the best reconstitution solvent in terms of response, considering the results of all experimental trials. The developed method for the estimation of zolpidem in human plasma was found to be simple, accurate, reproducible and highly sensitive within the range of 0.10 to 149.83 ng/mL. A thorough validation of analytical method for the assay in human plasma was carried out according to the USFDA guidelines (13), establishing the following validation parameters.

Preparation of required solutions

Preparation of stock solutions of analyte and IS

Primary stock solutions of zolpidem used for preparation of standard [calibration curve (CC)] and quality control (QC) samples were prepared from separate weighing. A primary stock solution of zolpidem (1 mg/mL) was prepared in methanol. The primary stock solution of IS (1 mg/mL) was prepared in methanol. The stock solutions of zolpidem and IS were stored at 2–8°C, and were found to be stable for 15 days. Intermediate neat standards and quality control samples were prepared separately from 1-mg drug stocks of CC and QC with Mill-Q water as intermediate dilution solvent, which was spiked to control plasma with 5% spiking. IS dilution was prepared at a 2.5 μg/mL concentration from 1-mg IS stock using Mill-Q water as diluent.

Preparation of calibration curve standards and QC samples

The linearity range was prepared from 0.10–149.83 ng/mL. $1/x^2$ regressions were used to estimate the amount of zolpidem in the subject plasma. Calibration samples and QC samples were prepared by spiking 50 μL of appropriate working solution of zolpidem into 950 μL of control human plasma. Four levels of zolpidem samples were prepared at concentrations of 0.10 [lower limit of quantification (LLOQ)], 0.30 [low quality control (LQC)], 68.88 [middle quality control (MQC)] and 113.85 ng/mL [high quality control (HQC)]. Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with zolpidem at appropriate concentrations and distributing 350-μL plasma aliquots into different tubes. All of the spiked samples were stored at −70°C.

To create the 20 mM ammonium formate buffer solution (w/v), approximately 1.26 gm of AR-grade ammonium formate was weighed and dissolved in 1,000 mL of Milli-Q water. The pH of the solution was adjusted to 5.00 ± 05 with GR-grade formic acid. To create the mobile phase (v/v), 75 parts of HPLC-grade methanol was mixed with 25 parts of 20 mm ammonium formate buffer and sonicated for 10 min. For drug stock dilution (10.000 μg/mL), 100 μL of drug stock (1 mg) was mixed with 9.900 mL of Milli-Q water.

Protocol for sample preparation

Before analysis, all frozen subject samples, calibration standards and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 300 μL of spiked plasma sample, 50 μL IS was added and vortexed for 20 s. To these samples, 2.5 mL of ethyl acetate was added and these samples were vortexed on a Vibramax (Spinx Instruments, Mumbai, India) at 2,500 rpm at 10°C for 10 min. Then the samples were centrifuged at 4,000 rpm for 10 min in a centrifuge (Refrigerated Centrifuge-Falcon 6/300 R, Sanyo) maintained at 10°C. Two milliliters of the supernatant was separated and evaporated to dryness under nitrogen at 50°C and 15 psi for 15 min. The dried samples were reconstituted with 300 μL of mobile phase and 10 μL was used for injection into the chromatographic system.

Method validation

Specificity and selectivity

The specificity of the method was evaluated by analyzing human plasma samples from six different lots to investigate the potential interferences at retention times of the analyte (zolpidem) and IS with an acceptance criteria of background noises, less than 20% response of the lowest standard curve point or LLOQ at the retention time of the zolpidem and less than 5% of the mean response of IS in LLOQ samples at retention time of IS. No significant interferences in the blank human plasma traces were found from endogenous components in drug-free human plasma at the retention times of the analyte and IS. Figure 4 shows the chromatograms of control blank plasma, blank plasma spiked with IS and LLOQ spiked with IS.

Calibration curve

After comparing the two weighting models, i.e., $1/x$ and $1/x^2$, a regression equation with a weighting factor of $1/x^2$ of analyte to IS concentration was found to produce the best fit for the concentration–detector response relationship for the analyte in human plasma for the linearity range of 0.10–149.83 ng/mL. By using the recommended $1/x^2$ model, values for correlation coefficient (r^2) were found to be ≥0.99, which indicates linearity over the whole calibration range for the analyte. The acceptance limit of accuracy for each of the back-calculated concentrations was ± 15%, except for LLOQ, where it was ± 20% (13). The samples were run in the order from low to high concentration. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. For a calibration run to be accepted, at least 75% of the standards, including the LLOQ and upper limit of quantification (ULOQ), were required to meet the acceptance criterion, otherwise the calibration curve was rejected. Figure 3B Shows the calibration curve of zolpidem.

Precision and accuracy

The intra-assay and inter-assay precision and accuracy were estimated by analyzing six replicates containing zolpidem at

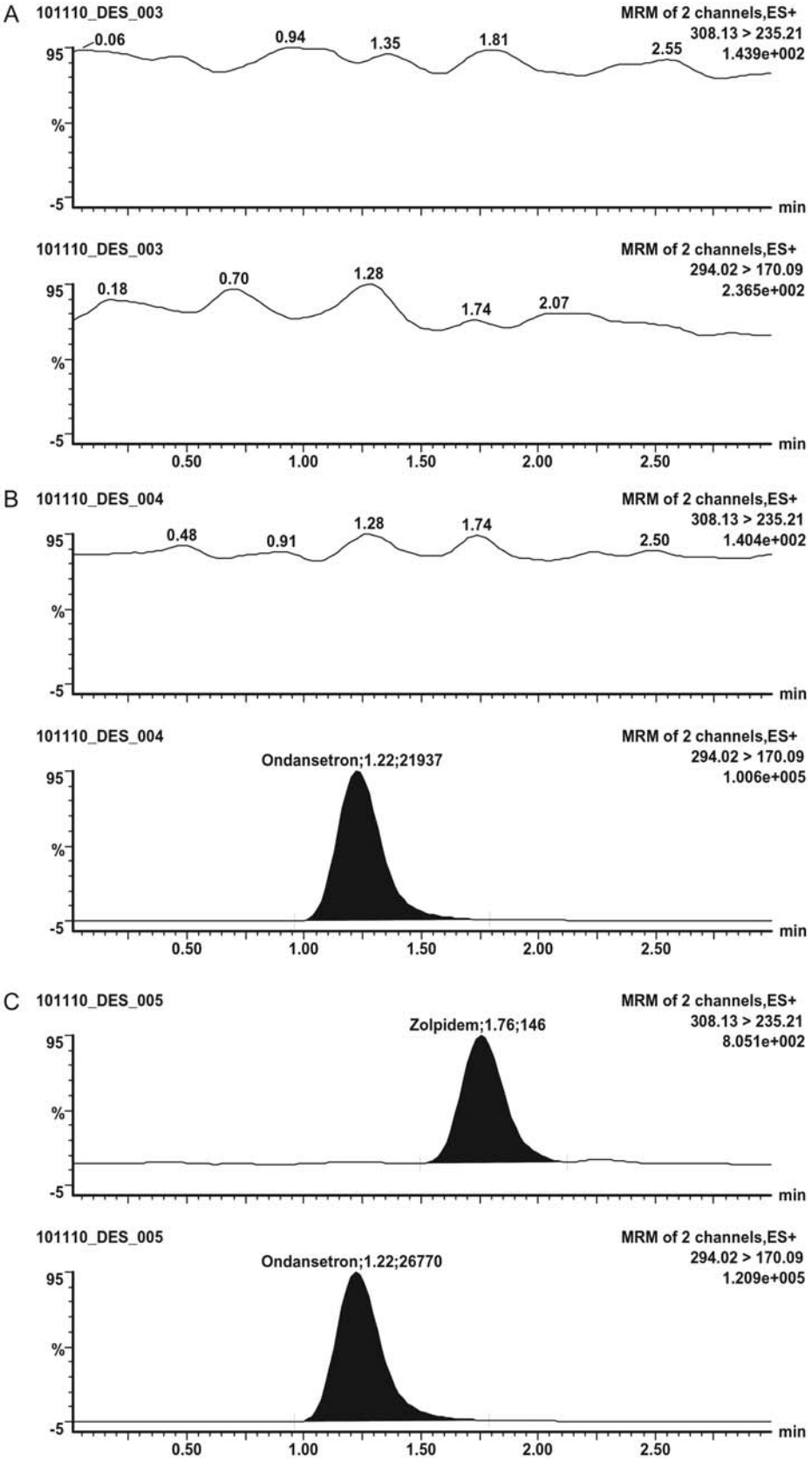

Figure 4. Typical MRM chromatograms of zolpidem (upper panel) and IS (lower panel) in: human blank plasma (A); human plasma spiked with IS (B); LLOQ sample along with IS (C).

Table II
Intra-and Inter-Day Precision and Accuracy of Zolpidem in Human Plasma*

QC	Run	Measured concentration of zolpidem (ng/mL)			
		Mean	SD	%CV	% Nominal
Intra-day and Inter-day variation (six replicates at each concentration)					
LLOQ (0.10 ng/mL)	1	0.10	0.007	6.96	101.67
	2	0.10	0.009	8.66	103.33
	3	0.10	0.008	8.66	96.67
	4	0.10	0.005	5.39	101.67
LQC (0.30 ng/mL)	1	0.31	0.013	4.27	101.67
	2	0.30	0.013	4.52	98.89
	3	0.32	0.016	4.99	105.56
	4	0.32	0.008	2.63	106.11
MQC (68.88 ng/mL)	1	67.81	2.075	3.06	98.44
	2	65.96	2.932	4.45	95.77
	3	66.58	3.693	5.55	96.66
	4	67.34	1.482	2.20	97.76
HQC (113.85 ng/mL)	1	113.35	4.002	3.53	99.56
	2	111.85	1.794	1.60	98.25
	3	112.29	0.583	0.52	98.63
	4	112.43	2.946	2.62	98.75
LLOQ		0.10	0.007	7.42	100.84
LQC		0.31	0.013	4.10	103.06
MQC1		66.92	2.546	3.81	97.16
HQC		112.48	2.331	2.07	98.80

*Note: Runs 01–03 are intra-day and Run 4 is inter-day.

four different QC levels; *viz*, LLOQ, LQC, MQC and HQC in human plasma. The acceptance criteria included accuracy within $\pm 15\%$ deviation from the nominal values, except LLOQ QC, where it should be $\pm 20\%$ and precision of $\leq 15\%$ relative standard deviation (RSD), except for LLOQ QC, where it should be $\leq 20\%$ (13). Both inter-day and intra-day experiments were highly accurate and precise. Table II shows the intra-and inter-batch precision and accuracy for zolpidem.

Ruggedness

Method ruggedness was determined by running a precision and accuracy batch processed by different analysts and using different Inertsil-ODS (4.6×50 mm) columns on a different Waters Acquity UPLC interfaced with Waters Quattro Micro API. Additionally, the method was tested for ruggedness by using a Waters instrument (Waters Alliance 2695 interfaced with Waters Quattro Micro API). Both experiments were accepted with the nominal percentage of six replicates for four levels of QCs.

Ruggedness was performed on a Waters 2695 HPLC system consisting of a Waters 2695 Separations Module with column heater and degasser, 2695 pump and thermostatted autosampler with heating and cooling. The separation of zolpidem and ondansetron was performed on an Inertsil-ODS, 3 V (4.6×50 mm, 5 μm) column that was maintained at 35°C in the column oven. Sample temperature was maintained at 10°C and the pressure of the system was 500 psi.

Simultaneous evaluation of matrix effect, recovery and process efficiency

Matrix effect, recovery and process efficiency for zolpidem were evaluated with aqueous (neat) samples, post-extracted and extracted samples from six different EDTA blank plasma lots. The results were calculated using peak area responses.

Matrix effect

Matrix effect was performed by analyzing six replicates of post extracted samples at five different CC standards; i.e., STD-1, STD-3, STD-5, STD-6 and STD-8 concentrations (prepared by spiking aqueous solutions into extracted blank plasma samples from six different blank EDTA plasma lots) along with six replicates of equivalent, similarly prepared aqueous (neat) samples. The percentage matrix effect was determined by comparing the mean peak area responses of post-extracted samples with mean peak area responses of aqueous (neat) samples at five different CC standards; i.e., STD-1, STD-3, STD-5, STD-6 and STD-8 concentration levels.

The percent coefficient of variation (%CV) and percent matrix effect on zolpidem in EDTA plasma ranged from 1.51 to 5.89% and 94.65 to 102.00, respectively. The %CV and percent matrix effect on ondansetron in EDTA plasma ranged from 1.79 to 4.42% and 98.15 to 102.46%, respectively.

Recovery

Recovery was performed by analyzing six replicates of extracted CC standard samples (spiked in six different blank EDTA plasma lots) along with post-extracted CC standard samples (prepared by spiking aqueous solutions into extracted blank plasma samples from six different blank EDTA plasma lots) at five different CC standard concentration levels; i.e., STD-1, STD-3, STD-5, STD-6 and STD-8. The percentage recovery was determined by comparing the areas of the extracted QC samples against equivalent post-extracted QC samples at five different CC standards; i.e., STD-1, STD-3, STD-5, STD-6 and STD-8 concentration levels. Recovery of zolpidem ranged from 82.12 to 91.63% and recovery of ondansetron ranged from 79.40 to 84.07%.

Process efficiency

Process efficiency was performed by analyzing six replicates of extracted CC standard samples (spiked in six different blank EDTA plasma lots) along with six replicates of equivalent, similarly prepared aqueous (neat) samples. The percentage process efficiency was determined by comparing the areas of the extracted samples against equivalent aqueous samples (neat samples) at five different CC standards; i.e., STD-1, STD-3, STD-5, STD-6 and STD-8 concentration levels. Process efficiency for zolpidem ranged from 81.32 to 89.61%, and from 80.32 to 83.10% for ondansetron. Table III shows the matrix effect, recovery and process efficiency for zolpidem and ondansetron IS in human plasma.

Dilution integrity

The dilution integrity exercise was performed with the aim of validating the dilution test to be carried out on higher analyte concentrations above the ULOQ during real-time analysis of subject samples. The dilution integrity experiment was carried out at 2.0 times the ULOQ concentration for the analyte. Six replicates each of half and quarter concentrations were prepared by 2-times and 4-times dilution with blank plasma and their concentrations were calculated by applying the dilution factors 2 and 4. The result of this experiment showed that ULOQ could be extended to 300.00 ng/mL for zolpidem. The

Table III
Matrix effect, Recovery and Process Efficiency for Zolpidem and Ondansetron Evaluated with Aqueous (Neat) Samples, Post-Extracted and Extracted from Six Different EDTA Blank Plasma Lots

Standard ID	Analyte Concentration (ng/mL)	Number of samples	Analyte peak response (zolpidem data)								
			Aqueous samples (neat)			Post-extracted samples			Extracted samples		
			Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Standard-1	0.10	$N = 6$	106	1.8	1.73	104	3.4	3.28	95	3.0	3.16
Standard-3	1.00	$N = 6$	1,048	61.7	5.89	992	15.7	1.58	885	13.6	1.54
Standard-5	50.01	$N = 6$	50,718	752.2	1.48	50,005	1,267.1	2.53	42,991	1,523.6	3.54
Standard-6	100.03	$N = 6$	84,431	1,933.3	2.29	83,603	2,242.7	2.68	68,656	1,495.7	2.18
Standard-8	149.83	$N = 6$	133,396	2,073.8	1.55	133,400	2,015.0	1.51	114,848	9,483.2	8.26

Standard ID	Analyte Concentration (ng/mL)	Number of samples	IS peak response (ondansetron data)								
			Aqueous samples (neat)			Post-extracted samples			Extracted samples		
			Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Standard-1	0.10	$N = 6$	26,973	993.0	3.68	27,512	493.6	1.79	22,414	621.8	2.77
Standard-3	1.00	$N = 6$	26,516	1,173.2	4.42	27,169	983.2	3.62	21,572	837.5	3.88
Standard-5	50.01	$N = 6$	27,197	885.5	3.26	26,796	984.2	3.67	21,843	528.3	2.42
Standard-6	100.03	$N = 6$	26,906	930.0	3.46	26,408	651.1	2.47	22,201	1,149.4	5.18
Standard-8	149.83	$N = 6$	26,509	917.4	3.46	26,188	953.7	3.64	21,355	628.5	2.94

Standard ID	Analyte Concentration (ng/mL)	Number of samples	% Matrix effect*			% Recovery†			% Process efficiency‡	
			Analyte	IS		Analyte	IS		Analyte	IS
Standard-1	0.10	$N = 6$	97.80	102.00		91.63	81.47		89.61	83.10
Standard-3	1.00	$N = 6$	94.65	102.46		89.16	79.40		84.39	81.35
Standard-5	50.01	$N = 6$	98.59	98.53		85.97	81.52		84.76	80.32
Standard-6	100.03	$N = 6$	99.02	98.15		82.12	84.07		81.32	82.52
Standard-8	149.83	$N = 6$	100.00	98.79		86.09	81.54		86.10	80.56
					Mean	87.00	81.60	Mean	85.23	81.57
					SD	3.597	1.657	SD	3.006	1.212
					%CV	4.13	2.03	%CV	3.53	1.49

*%Matrix effect: post-extracted mean response / aqueous (neat) mean response × 100.
†%Recovery: extracted mean response / post-extracted mean response × 100.
‡%Process efficiency: extracted mean response / aqueous mean response × 100.

mean backcalculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal with a %CVs of 100.60% and 94.17%, respectively.

Stability experiments

The stability of zolpidem and IS in the injection solvent was determined by injecting replicate preparations of processed plasma samples for up to 45.18 h (in the autosampler at 10°C) after the sample loading. Wet extract stability was successfully assessed by analyzing six replicates of wet extract stability samples stored at a temperature below 10°C for 44.32 h at low and high concentrations. The stability of the analyte (zolpidem) in plasma within 5.00 h (bench-top) was determined at ambient temperature (~25°C) at two concentrations (LQC and HQC) in six replicates. The stability of zolpidem in human plasma following five freeze-thaw cycles was assessed, in which the samples were stored at −70°C between freeze/thaw cycles thawed by allowing them to stand (unassisted) at room temperature for ~2.5 h and then returned to the freezer. Freezer stability (long-term) of the analyte in human plasma was assessed by analyzing the LQC and HQC samples stored at −70°C using the same procedure as described in previously. Samples were considered stable if assay values were within the acceptable limits of accuracy (±15%) and precision (≤15% RSD or %CV). Results of the stability experiments are shown in Table IV.

Pharmacokinetic study

The design of the study consisted of a randomized open label, balanced, single dose two-treatment, two-sequence, two-period, comparative oral bioavailability study of 10-mg tablets of zolpidem tartrate under fasting conditions. Each volunteer was judged to be in good health through medical history, physical examination and routine laboratory examination. Written consent was taken from all volunteers after informing them about the objectives and possible risks involved in the study.

Table IV
Stability Data of Quality Controls in Human EDTA Plasma for Zolpidem

QC ID	Type of stability	Mean	SD	%CV	% Nominal	% Change
LQC (0.30 ng/mL)	Bench-top (5.00 h)	0.30	0.007	2.48	99.22	0.78
	Injector (45.18 h)	0.29	0.021	7.41	96.11	3.89
	Wet extract (44.32 h)	0.30	0.024	7.89	100.00	0.00
	Freeze and thaw (5 cycles)	0.30	0.012	3.99	101.11	−1.11
	LT stability in EDTA plasma (126.71 days)	0.31	0.014	4.46	102.22	−2.22
HQC (113.85 ng/mL)	Bench-top (5.00 h)	112.10	2.972	2.65	98.47	1.53
	Injector (45.18 h)	116.55	3.253	2.79	102.38	−2.38
	Wet extract (44.32 h)	112.12	3.157	2.82	98.48	1.52
	Freeze and thaw (5 cycles)	115.52	3.176	4.48	101.47	−1.47
	LT stability in EDTA plasma (126.71 days)	112.11	1.352	1.21	98.47	1.53

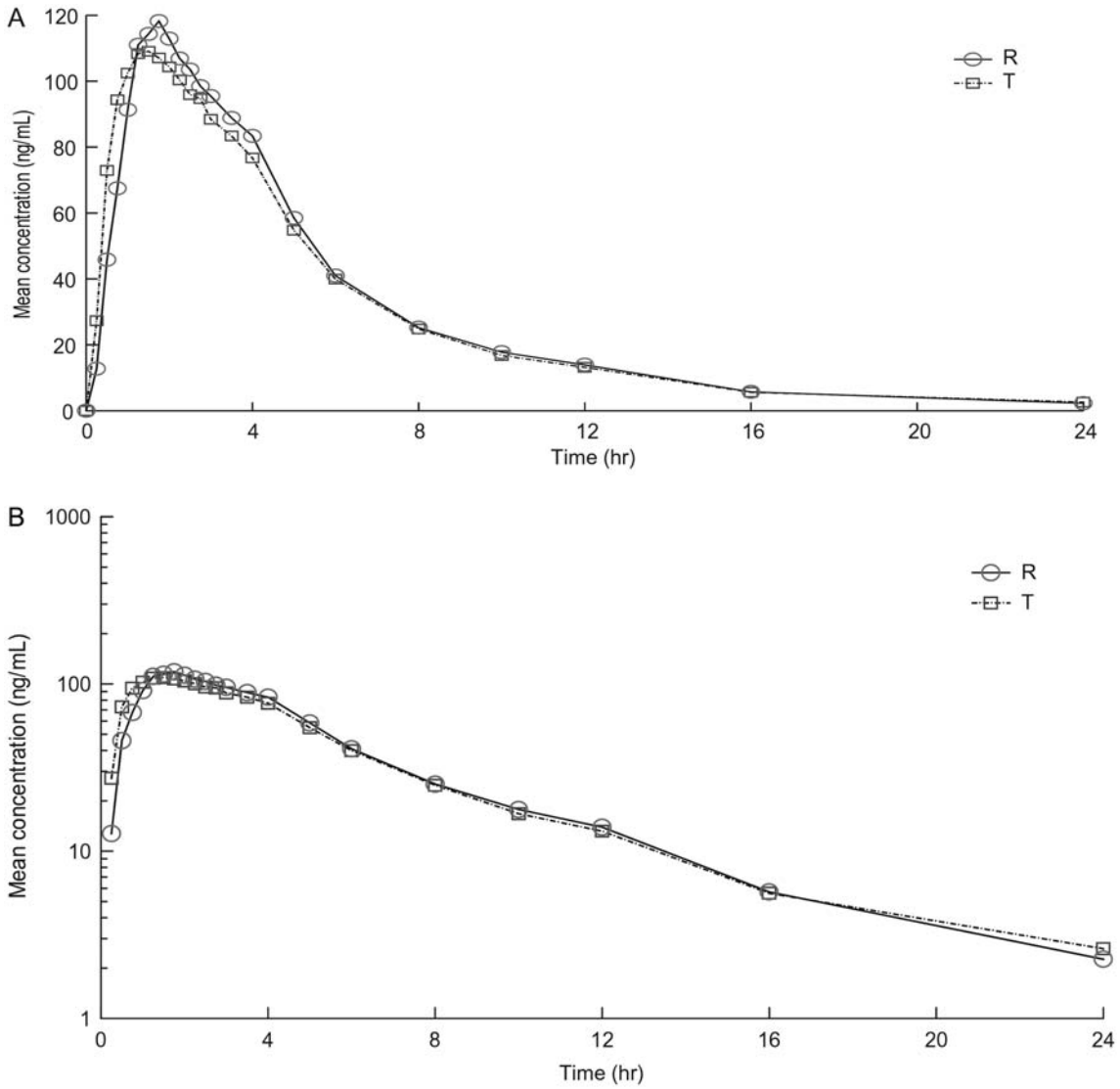

Figure 5. Linear plot of mean plasma zolpidem concentrations versus time in healthy, adult, male human subjects under fed conditions (A); semi-log plot of mean plasma zolpidem concentrations versus time in healthy, adult, male human subjects (B).

An independent ethics committee constituted as per the Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA (14). To verify the sensitivity and selectivity of this method in a real-world situation, a pharmacokinetic study was conducted on healthy volunteers ($n=8$) following oral administration of 10 mg zolpidem. Blood samples were collected before dosing and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00 and 24.00, using K2EDTA vacationer collection tubes (BD, Franklin, NJ). The tubes were centrifuged at 2,500 rpm, 4°C for 10 min and the plasma was collected. The collected plasma samples were stored at −70°C until use. Along with clinical samples, QC samples at low, middle and high concentrations were assayed in duplicate and distributed among the unknown samples in the analytical run using the previously validated procedure. The analytical runs were accepted if no more than 33% of the QC samples were greater than ± 15% of the nominal concentration. The plasma concentration−time profile of zolpidem was analyzed by the non-compartmental method using WinNonlin Version 5.2 (Pharsight Corporation, Mountain View, CA). The median T_{max} values for Zolpidem in 10-mg tablets (test and reference) were 1.25 and 1.75 h, respectively. The 90% confidence intervals for test and reference zolpidem tartrate 10 mg tablets for Ln-transformed parameters C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ were 93.78−103.89%, 90.38−103.43% and 90.27−103.81%, respectively. The pharmacokinetic parameters of the test and reference zolpidem tartrate 10-mg tablets were within the 80−125% acceptance range. Figure 5A shows the AUC and con versus time plot of zolpidem for both reference and test samples conducted in the pharmacokinetic study, and Figure 5B shows the semi-log plot of mean plasma zolpidem concentrations versus time in healthy, adult male human subjects.

Conclusion

In summary, we have developed and validated a selective, reproducible and high-throughput LC–MS-MS method to quantify zolpidem using ondansetron as IS. To the best of our knowledge, the cost effectiveness, simplicity of the liquid–liquid extraction assay and sample turnover rate of less than 3.00 min per sample made it possible to analyze more than 350 plasma samples per day in high-throughput bioanalysis of zolpidem. As discussed, the method is highly rugged and can be used in an HPLC system. The method was proved to be reproducible with a good recovery and minimum matrix effect. At five freeze-thaw cycles, stock solutions were stable at room temperature for at least 15 days. In LC–MS-MS analytical module and from the results of the validation parameters, we can conclude that the developed method can be useful for Bioavailability and Bioequivalence studies and routine therapeutic drug monitoring with desired precision and accuracy.

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