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HPLC–ESI-MS/MS validated method for simultaneous quantification of zopiclone and its metabolites, N-desmethyl zopiclone and zopiclone-N-oxide in human plasma

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Abstract

A simple, selective and sensitive isocratic HPLC method with triple quadrupole mass spectrometry detection has been developed and validated for simultaneous quantification of zopiclone and its metabolites in human plasma. The analytes were extracted using solid phase extraction, separated on Symmetry shield RP8 column (150 mm × 4.6 mm i.d., 3.5 μm particle size) and detected by tandem mass spectrometry with a turbo ion spray interface. Metaxalone was used as an internal standard. The method had a chromatographic run time of 4.5 min and linear calibration curves over the concentration range of 0.5–150 ng/mL for both zopiclone and N-desmethyl zopiclone and 1–150 ng/mL for zopiclone-N-oxide. The intra-batch and inter-batch accuracy and precision evaluated at lower limit of quantification and quality control levels were within 89.5–109.1% and 3.0–14.7%, respectively, for all the analytes. The recoveries calculated for the analytes and internal standard were ≥90% from spiked plasma samples. The validated method was successfully employed for a comparative bioavailability study after oral administration of 7.5 mg zopiclone (test and reference) to 16 healthy volunteers under fasted condition.

Keywords: Zopiclone; N-desmethyl zopiclone; Zopiclone-N-oxide; LC–MS/MS; Solid phase extraction

1. Introduction

Zopiclone, a cyclopyrrolone derivative, is a short-acting hypnosedative used in the treatment of insomnia. It belongs to a novel chemical class which is structurally unrelated to existing hypnotics [1]. However, the pharmacological profile of zopiclone is similar to that of the benzodiazepines. It possesses a chiral centre and is commercially available as a racemic mixture of two stereoisomers, only one of which is active—the eszopiclone [2]. At the clinically recommended dose of 7.5 mg zopiclone, peak plasma concentration of 60 ng/mL is achieved within 90 min with bioavailability greater than 80%, indicating the absence of a significant first-pass effect. The plasma protein binding of zopiclone has been reported to be between 45% and 80% [3]. Zopiclone is rapidly and widely distributed to body tissues including the brain, and is excreted in urine, saliva and breast milk. It is extensively metabolized by three major pathways. These metabolic pathways include oxidation to an active zopiclone-N-oxide (∼12%), demethylation to inactive N-desmethyl zopiclone (∼16%) and oxidative decarboxylation to various other metabolites. The N-oxide and N-desmethyl analogues are excreted renally; the products of decarboxylation are partly eliminated via lungs as carbon dioxide. Only the N-oxide analogue has weak pharmacological activity [4,5].

As hypnotics and benzodiazepines are routinely screened for clinical and forensic toxicology applications, several analytical procedures are reported to determine zopiclone and/or its metabolites in a variety of biological matrices like, whole blood [6–10], plasma [11–23], serum [10,24–27], urine [7,10,14,23,28–36], hairs [7,37], nails [37], oral fluids [38,39] and others [40]. These methods have been developed employing different analytical techniques viz. capillary electrophoresis [28], radioimmunoassay [33,35], gas chromatography [8,11,15,20,27], gas chromatography–mass spectrometry [6,8,30,40], liquid chromatography–mass spectrometry [7,10,17,24,29,31,37–39], high performance liquid

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chromatography with UV [9,13,18,19,22,25,32], diode array [21,40] and fluorescence detection [10,12,14,16,36]. Majority of these methods are for the detection and quantitation of zopiclone in combination with other benzodiazepines. However, very few methods are reported for the simultaneous determination of zopiclone and its metabolites in human plasma with desired sensitivity and selectivity for bioavailability/bioequivalence studies.

LeLiboux et al. [14] have determined zopiclone and its two major metabolites in plasma and urine by HPLC with fluorescence detection. The limit of detection of the method was 5 ng/mL for zopiclone and 10 ng/mL for its metabolites. An enantioselective determination of zopiclone and its metabolites in urine by capillary electrophoresis was given by Hempel and Blaschke [28]. This method was comparatively faster and simpler than chiral LC, employing liquid–liquid extraction with chloroform–isopropanol mixture. However, the sensitivity of the method was a limiting factor. Another method for chromatographic screening of zopiclone and its metabolites in urine using HPLC and LC–MS was demonstrated by Nordgren et al. [32]. The sensitivity and chromatographic run time of this method were not favourable for routine subject sample analysis. A method based on HPLC-fluorescence has been presented by Gupta [10] for the simultaneous determination of zopiclone and its two active metabolites (N-oxide and N-desmethyl) in human blood, serum and urine. However, the sensitivity and overall analysis time were its limiting factors. A highly sensitive LC–MS methodology has been proposed by Tornio et al. [23] for zopiclone (0.025 ng/mL) and its active metabolites (0.1 ng/mL) in human plasma and urine with a long chromatographic run time. But the paper does not mention the extraction procedure employed and the plasma volume used for processing, which are critical in assessing the applicability of this method for subject sample analysis. Recently, Nirogi et al. [16] have developed and validated a method for simultaneous determination of zopiclone and its metabolite N-desmethyl zopiclone by HPLC-fluorescence. The linear dynamic range was 3–300 and 6–500 ng/mL for zopiclone and its metabolite, respectively. The run time of this method was too long (13 min), which could be a drawback when large number of samples needs to be quantified.

Thus, the objective of the present study was to develop and validate a sensitive and fast LC–MS/MS method for simultaneous quantitation of zopiclone and its metabolites in human plasma for routine sample analysis. The extraction procedure employed is highly efficient and gives reproducible recoveries for all the analytes as well as the internal standard. The method presented is simple, selective and sensitive to support a pharmacokinetic study of zopiclone and its metabolites for the recommended dose of 7.5 mg zopiclone in human subjects.

2. Experimental

2.1. Chemicals and materials

Reference standards of zopiclone (ZOP), N-desmethyl zopiclone (NDZOP) and zopiclone-N-oxide (ZOPNO) were provided by Ipca Ltd. (Mumbai, India). Metaxalone (IS) was obtained from Farchemia, SRL (Mumbai, India). All these standards had purity ≥99%. HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Formic acid of AR grade was procured from Qualigens Ltd. (Mumbai, India). Purified water was generated from Milli Q A10 gradient water purification system purchased from Millipore (Bangalore, India). Blank human blood was collected with heparin from healthy and drug free volunteers. After centrifugation at 2680 × *g* at room temperature, plasma was collected and stored at −20 °C. Orpheous DVB-HL (30 mg, 1 mL) solid phase extraction cartridges were procured from Orochem India Pvt. Ltd. (Mumbai, India).

2.2. Liquid chromatography and mass spectrometric conditions

An API-2000 LC–MS/MS triple quadrupole mass spectrometer equipped with a Turbo Ion Spray™ ionization source (Applied Biosystems/MDS Sciex, Toronto, Canada) was used for tandem mass spectrometry. MS/MS analysis was performed in multiple reaction monitoring (MRM) and positive ionization mode, using mass transitions of *m/z* 389.1 → 245.1 for ZOP; *m/z* 375.0 → 245.1 for NDZOP; *m/z* 405.1 → 245.1 for ZOPNO; and *m/z* 222.1 → 161.0 for metaxalone (IS). Figs. 1–4 show the mass spectra of parent and product ions for analytes and IS, respectively. The source dependant parameters optimized were nebuliser gas (gas 1): 25 psig, heater gas (gas 2): 75 psig, curtain gas: 12 psig, source temperature: 400 °C. CAD gas was set at 3 psig. Compound dependant parameters set were declustering potential (DP): 42, 24, 10 and 38; collision energy (CE): 24, 30, 27 and 18; and cell exit potential (CXP): 7, 8, 7 and 10 for ZOP, NDZOP, ZOPNO and IS, respectively. Entrance potential (EP) and focusing potential (FP) were kept at 10 and 400 V, respectively. Ion spray voltage (ISV) was set at 5200 V. Q1 and Q3 were maintained at unit resolution. Dwell time was kept at 300 ms and no cross talk was found between transitions. Peak areas were integrated using analyst software version 1.4. Linear regression with weighing of 1/*x* was used to construct the standard curves.

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of binary LC-20AD prominence pump, autosampler (SIL-HTc), solvent degasser (DGU-20A_3 prominence) and temperature controlled compartment for column (CTO 10AVP). The analytical column, Symmetry shield RP8 (150 mm × 4.6 mm i.d., 3.5 μm particle size) from Waters (India) Pvt. Ltd. (Bangalore, India) was used for separation of analytes and IS. The flow rate of the mobile phase under isocratic condition was kept at 0.5 mL/min, with 60% of the eluate directed to MS and 40% to waste. The auto sampler temperature was set at 10 °C and the injection volume was 5 μL. The mobile phase consisted of 0.05% formic acid in water:acetonitrile:methanol (25:65:10, v/v/v). The column oven temperature was maintained at 25 °C and the total LC run time was 4.5 min.

2.3. Standard and quality control preparation

The standard stock solutions of ZOP, NDZOP and ZOPNO were prepared by dissolving their accurately weighted com-

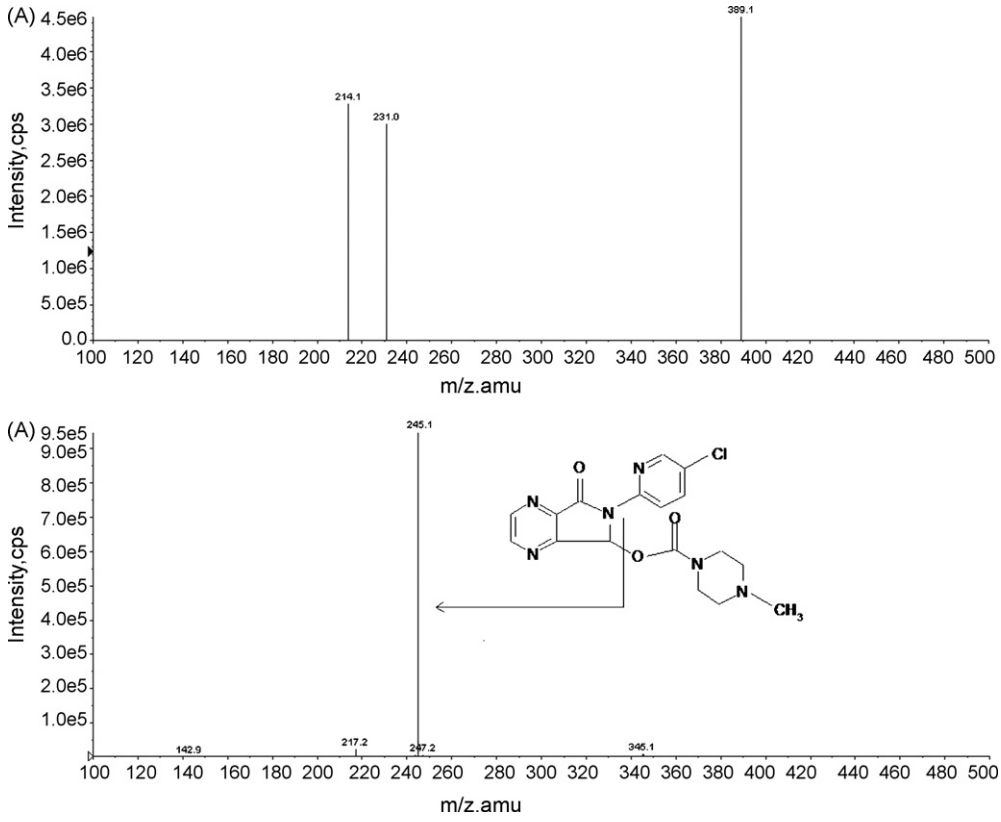

Fig. 1. Precursor ion (A) and product ion spectra (B) of zopiclone.

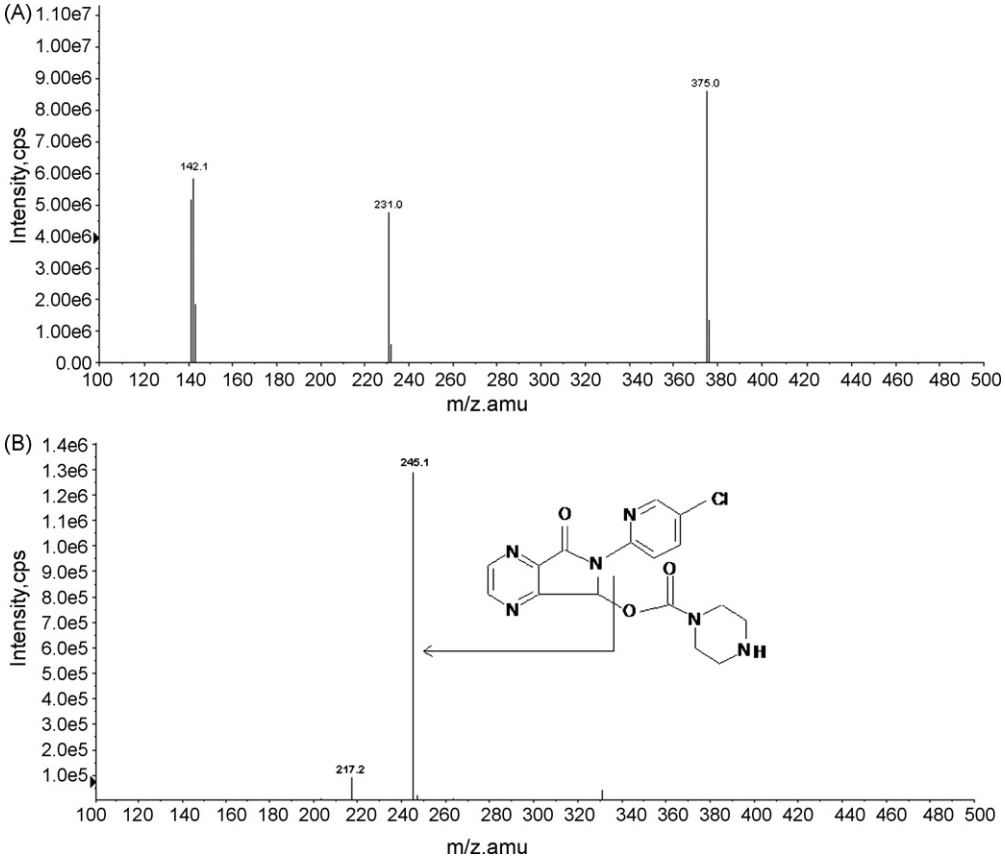

Fig. 2. Precursor ion (A) and product ion spectra (B) of N-desmethyl zopiclone.

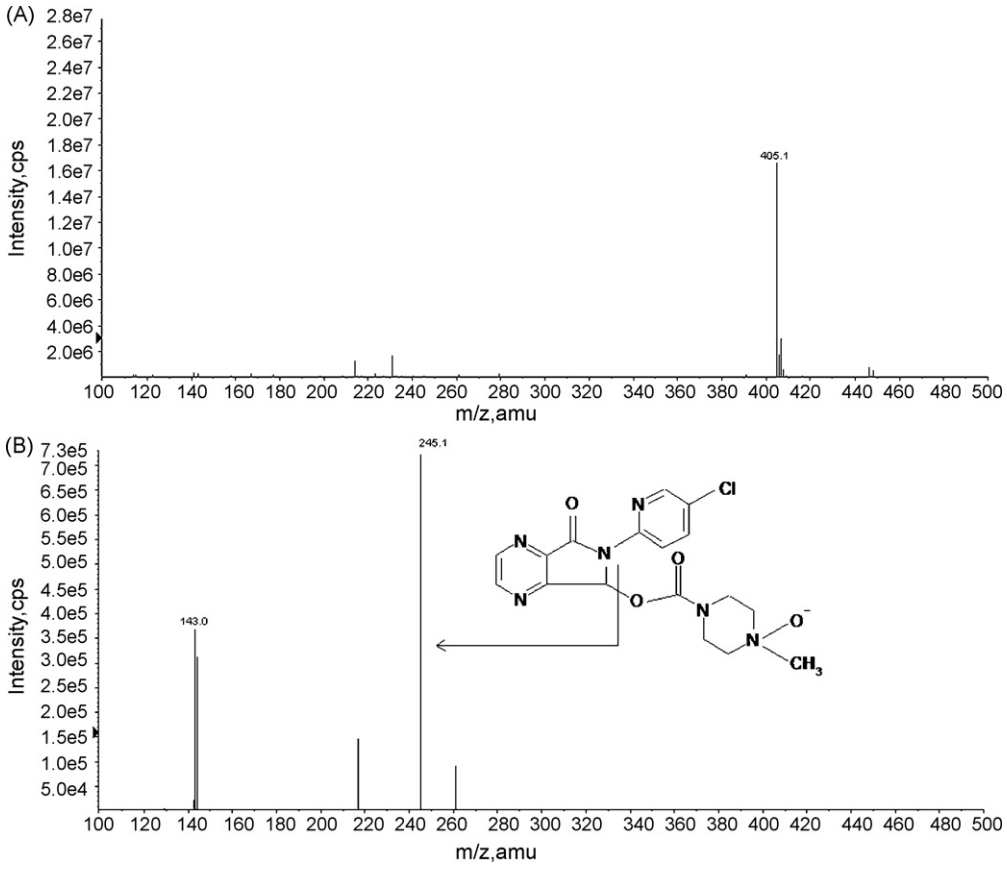

Fig. 3. Precursor ion (A) and product ion spectra (B) of zopiclone-N-oxide.

pounds in acetonitrile:water (60:40, v/v), while metaxalone was dissolved in methanol to give a final concentration of 1000 μg/mL. The standard stock solutions of analytes were then serially diluted with acetonitrile:water (60:40, v/v) to obtain working solutions of required concentrations. All the solutions were stored at 2–8 °C and were brought to room temperature before use.

The calibration standards and quality control (QC) samples were prepared by spiking (5% of the total plasma volume) with working solutions. Calibration samples were prepared at concentrations of 0.5, 1, 2, 5, 20, 60, 100 and 150 ng/mL for ZOP and NDZOP while 1, 1.5, 2, 5, 20, 60, 100 and 150 ng/mL for ZOPNO. Quality control samples were prepared at 0.5 ng/mL (lower limit of quantification, LLOQ), 1.5 ng/mL (low quality control, LQC), 50 ng/mL (medium quality control, MQC) and 137.5 ng/mL (high quality control, HQC) for ZOP and NDZOP while 1 ng/mL (LLOQ), 3 ng/mL (LQC), 50 ng/mL (MQC) and 137.5 ng/mL (HQC) for ZOPNO. Aliquots of spiked plasma samples were taken in micro centrifuge tubes and stored at −20 °C.

2.4. Sample preparation

All frozen subject samples, calibration standards and quality control samples were thawed at room temperature prior to analysis. The samples were adequately vortexed and centrifuged at 1700 × *g* for 5 min at 10 °C to settle any solid present that may block the cartridge. An aliquot of 0.5 mL plasma sample was mixed with 50 μL of internal standard (5.0 μg/mL) and 0.5 mL, 0.05% (v/v) formic acid in water. This sample mixture was loaded on DVB-HLB cartridges, previously conditioned with 1 mL of methanol followed by 1 mL, 0.05% (v/v) formic acid in water. The extraction cartridges were washed with 1 mL of 0.05% (v/v) formic acid followed by 1 mL of 10% methanol. Elution was carried out with 1 mL of 0.1% (v/v) formic acid in acetonitrile. The eluate was then evaporated to dryness at 40 °C. The dry residue was dissolved in 300 μL of mobile phase and 5 μL was used for injection in the LC–MS/MS system.

2.5. Method validation

The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, stability and dilution integrity according to USFDA guidelines [41].

Selectivity of the method was assessed in two sets of plasma, each set comprised of 10 heparinised blank plasma out of which 8 were normal, 1 was haemolysed and the other was lipemic plasma. In the first set, plasmas were extracted and directly injected for LC–MS/MS detection. In the other set, blank plasmas were spiked with LLOQ working solution of analytes, extracted and analysed in the similar manner. The second set was also used for sensitivity determination.

The linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibra-

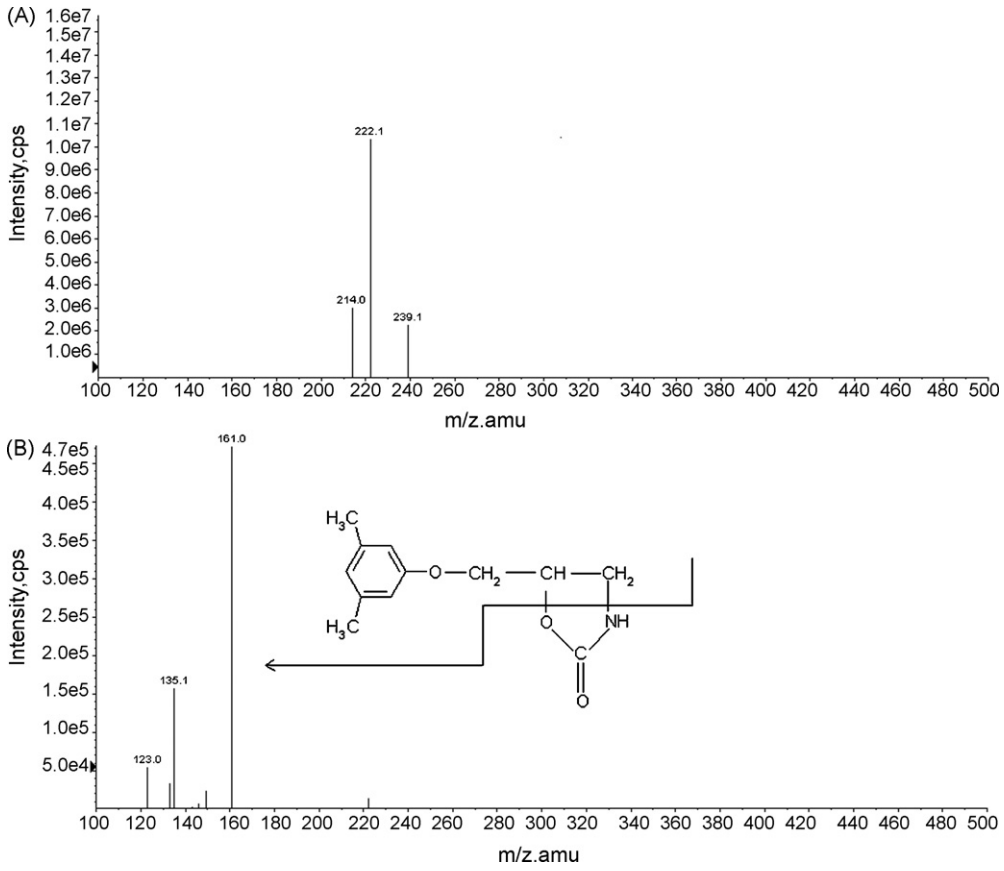

Fig. 4. Precursor ion (A) and product ion spectra (B) of metaxalone (IS).

tion curve. Twelve linearity curves containing eight non-zero concentrations were analysed. The calculation was based on the peak area ratio of analyte versus internal standard. The peak area ratio values of calibration standards were proportional to the concentrations of the analytes in plasma over the ranges tested.

Intra-batch and inter-batch accuracy and precision of the method were assessed at four different concentrations (LLOQ, LQC, MQC and HQC) for each analyte. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision in terms of relative error (%RE) and coefficient of variation (%CV), respectively, were calculated.

Recovery presents the extraction efficiency of a method and was performed at LQC, MQC and HQC levels. It was evaluated by comparing peak area of extracted samples to the peak area of unextracted samples (quality control working solutions spiked in to reconstitution solution).

Stability experiments were performed to evaluate the analyte stability in stock solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability samples of analytes and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze–thaw stability, evaporation and long-term stability were performed at LQC and MQC level using six replicates at each level. To evaluate the evaporation stability, samples were kept for evaporation of eluent in a solvent evaporator for 20 min. Further, it was kept for 1 h in the evaporator and the stability was compared with freshly prepared samples at identical concentrations for both the QC levels. This stability was performed to check any loss in assay due to excessive evaporation.

Ion suppression or enhancement due to matrix components was checked in six plasma samples by injecting the unextracted MQC (spiked externally with working solution of MQC in extracted plasma) and pure aqueous MQC samples. The area responses of both the injections were compared for all the analytes. Furthermore, six different lots (four normal, one haemolysed and one lipemic) were taken and three LQCs and three HQCs were prepared from each lot by adding externally, respective working solution of analytes and IS in extracted blank plasma. %CV of area ratio of analyte to IS at each level (LQC and HQC) was calculated. The effect of IS on ionization was also checked by injecting the unextracted MQC sample with and without IS addition.

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above upper limit of quantification, ULOQ), which may be encountered during real subject samples analysis. It was carried out at 1.5 times the ULOQ concentration. Six replicate samples of 1/2 and 1/4 concentrations were prepared and their concentrations were calculated, by applying the dilu-

tion factors of 2 and 4 against the freshly prepared calibration curve.

2.6. Application to a pharmacokinetic study

The proposed method was applied to a pharmacokinetic study in healthy volunteers. The design of study comprised of "A randomized, open label, single dose comparative bioavailability study of 7.5 mg zopiclone tablets in 16 normal healthy subjects under fasted condition". All the subjects were informed of the aim and risk involved in the study and informed consent were obtained. An ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [42]. Health check up for subjects was done by general physical examination, ECG and laboratory tests like haematology, biochemistry and urine examination. Also, the tests for HIV, HBSAg and HCV were negative in all the subjects. Each subject was orally administered a single dose of test and reference formulation with 240 mL of water. Their blood samples were collected in tubes containing heparin before and after 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.25, 02.50, 02.75, 03.00, 04.00, 05.00, 06.00, 08.00, 10.00, 12.00, 16.00, 24.00 and 36.00 h of administration of drug. The samples were centrifuged at 1700 × *g* for 10 min and plasma was separated, stored at −20 °C until use.

3. Results and discussion

3.1. Method development

The aim of this study was to develop and fully validate an LC–ESI-MS/MS method for simultaneous quantification of zopiclone and its metabolites in human plasma. The mass spectra of analytes were acquired by continuous infusion of standard solution (500 ng/mL) at 10 μL/min flow rate and scanning Q1 from *m/z* 100 to 500. The signal intensities observed in positive ion mode were much higher than those in negative ion mode. The signal intensities were further increased by acidifying the standard solutions with formic acid as there are number of potential sites for protonation. The ESI-MS spectra contained primarily the $[M+H]^+$ ion at *m/z* 389.1, 375.0 and 405.1 for zopiclone, N-desmethyl zopiclone and zopiclone-N-oxide, respectively. All precursor ions were fragmented using collision activated dissociation (CAD) gas and by applying sufficient collision energy. The most abundant product ions obtained were at *m/z* 245.1 for all the three analytes. The response found was adequate when Q1 and Q3 were set at unit and low resolution, respectively, but with significant background interference. When Q3 resolution was set at unit resolution, there was considerable increase in signal/noise (S/N) ratio with proportional decrease in noise level. Further, experiments were conducted by varying other parameters to reduce the background noise. Declustering potential, collision energy and CAD gas were suitably optimized to fragment solvent and other interfering ions. The optimized curtain gas helped in avoiding solvent ions from entering in the mass interface. A dwell time of 300 ms was sufficient to avert any cross talk among the fragment ions (*m/z* 245.1) of zopiclone, N-oxide zopiclone and N-desmethyl zopiclone used for quantification. As APCI source gave low intensity for the protonated parent ions even after optimizing nebuliser current, turbo ion spray (TIS) source was preferred in the present study.

Chromatographic separation of analytes and IS from matrix components helps in improving the selectivity of the method. To achieve this, columns (C8 and C18) of different lengths and particle sizes were tested. A short column with small particle size (3.5 μm) was sufficient to attain good peak shapes but was unable to remove interfering components which resulted in ion suppression. Thus, a long column (150 mm × 4.6 mm, 3.5 μm particle size) was preferred to minimize the interferences from matrix components. Different compositions of mobile phases were tried to get a good resolution with peak shape of analytes and IS in support of the long column selected. Use of 0.05% formic acid:acetonitrile (25:75, v/v) helped in obtaining symmetric peak shape for zopiclone, N-desmethyl zopiclone and IS, but not for zopiclone-N-oxide. A combination of 0.05% formic acid:acetonitrile:methanol in 25:65:10 (v/v/v) ratio as the mobile phase assisted in getting the desired selectivity as well as the peak shape for all the analytes and IS. Addition of methanol upto 10% (v/v) was sufficient to separate the interference peak from the peak of zopiclone-N-oxide. Small particle size column (3.5 μm) made the peaks sharper and smoother. The use of acetic acid in place of formic acid suppresses the ion signals and thus decreases their intensities. The flow rate was kept at 0.5 mL/min and only 60% of total flow was allowed to enter the interface, which helped to prevent excess solvent ions from entering the MS system. Each chromatographic run was completed within 4.5 min.

After finalizing the mobile phase, all three compounds along with IS were retuned with mobile phase to set nebuliser gas, heater gas, ion spray voltage (ISV) and source temperature. These were the source dependant parameters which further improved the intensity with higher signal/noise ratio.

To obtain cleaner plasma samples with sufficient recovery, different extraction techniques were investigated. Preliminary experiments were performed on hydrophilic–lipophilic balance cartridges, which gave consistent results in terms of recovery for all the analytes with IS. Liquid–liquid extraction was unable to give reproducible recovery using different organic solvents (hexane, methyl ter-butyl ether, diethyl ether and ethyl acetate) and their mixtures in different ratios. Also, it made the extraction procedure cumbersome. Precipitation using acetonitrile or methanol gave strong interferences.

Prior to SPE, the samples were acidified to break the protein–drug binding and maintain the analytes in an ionized form [43]. Washing of cartridges with 10% methanol was necessary to remove any non-polar interference. The elution was carried out with high formic acid content in acetonitrile, i.e. 1 mL of 0.1% formic acid in acetonitrile. Further, eluate was evaporated to dryness under nitrogen and reconstituted with 0.3 mL mobile phase to increase the response and make the injection solution compatible with mobile phase.

Ideally, the internal standard should mirror the analyte in as many ways as possible. Selection of metaxalone as internal standard in this study was appropriate as it was chromatograph-

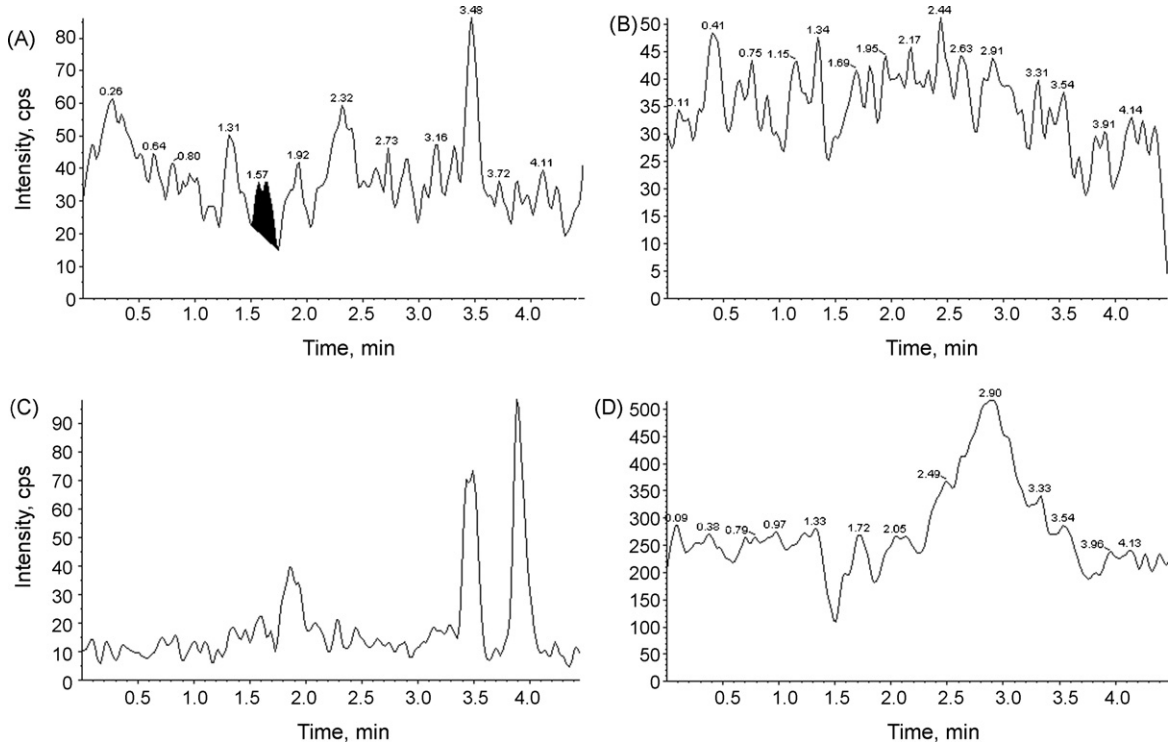

Fig. 5. Blank plasma chromatograms for (A) zopiclone (389.1/245.1), (B) N-desmethyl zopiclone (375.0/245.1), (C) zopiclone-N-oxide (405.1/245.1) and (D) metaxalone (222.1/161.0).

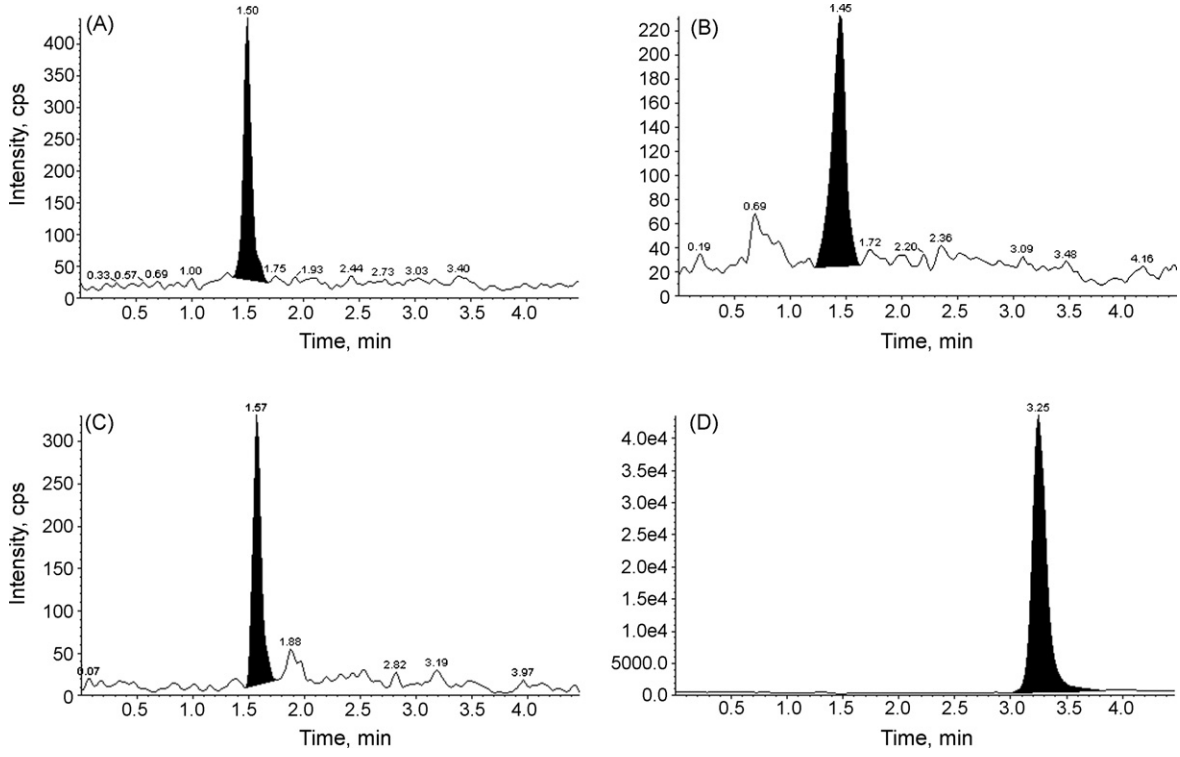

Fig. 6. Chromatograms at LLOQ for (A) zopiclone (389.1/245.1), (B) N-desmethyl zopiclone (375.0/245.1), (C) zopiclone-N-oxide (405.1/245.1) and (D) metaxalone (222.1/161.0).

ically well separated and its presence gave no ion suppression of analytes. Moreover, the results of method validation using metaxalone as the IS were acceptable in this study based on FDA guidelines.

3.2. Selectivity and sensitivity

Selectivity of the method towards the endogenous plasma matrix was evaluated by calculating signal (LLOQ) to

Table 1
Summary of calibration curve standards

	Zopiclone								Correlation coefficient[a] (r)	Slope[a]	Intercept[a]
Nominal concentration (ng/mL)	0.5	1.0	2.0	5.0	20.0	60.0	100.0	150.0	0.9993	0.00003	0.00798
Calculated concentration (ng/mL)	0.47	0.98	1.97	4.99	21.48	61.07	100.70	146.83			
%CV	7.5	8.1	5.1	4.5	2.5	2.6	3.2	2.4			
%RE	−5.5	−2.3	−1.5	−0.3	7.4	1.8	0.7	−2.1			
	N-desmethyl zopiclone								Correlation coefficient[a] (r)	Slope[a]	Intercept[a]
Nominal concentration (ng/mL)	0.5	1.0	2.0	5.0	20.0	60.0	100.0	150.0	0.9992	0.00005	0.00535
Calculated concentration (ng/mL)	0.49	1.00	1.96	4.94	21.18	61.08	101.38	146.48			
%CV	7.1	8.1	7.0	3.5	2.7	2.5	4.2	2.9			
%RE	−3.1	−0.5	−1.8	−1.3	5.9	1.8	1.9	−2.3			
	Zopiclone-N-oxide								Correlation coefficient[a] (r)	Slope[a]	Intercept[a]
Nominal concentration (ng/mL)	1.0	1.5	2.0	5.0	20.0	60.0	100.0	150.0	0.9994	0.00000	0.00264
Calculated concentration (ng/mL)	1.00	1.41	1.96	5.00	21.04	60.16	100.93	147.96			
%CV	6.8	10.6	6.0	5.3	3.4	3.5	3.7	2.1			
%RE	−0.9	−6.0	−2.1	0.0	5.2	0.3	0.9	−1.4			

CV: coefficient of variation; RE: relative error.
[a] Mean value.

noise (blank) ratio in 10 batches of plasma samples for the three analytes. The mean S/N ratio found was 19.55, 29.68 and 15.86 for ZOP, NDZOP and ZOPNO, respectively. Chromatograms for blank plasma and at LLOQ in Figs. 5 and 6 demonstrate the sensitivity and selectivity of the method with retention times of 1.50, 1.45, 1.57 and 3.25 min for ZOP, NDZOP, ZOPNO and IS, respectively. The mean accuracy for back calculated concentration of all three analytes was within 89–102% and %CV was less than 13%.

3.3. Linearity, accuracy and precision and recovery

Calibration curves were linear from 0.5 to 150 ng/mL with correlation coefficient $r \geq 0.9992$ for ZOP and NDZOP; from 1 to 150 ng/mL with $r \geq 0.9994$ for ZOPNO. The correlation coefficient (r) values, slopes and intercepts were calculated using weighted ($1/x$) linear regression analysis. The observed mean back calculated concentration with accuracy (%RE) and precision (%CV) of 12 curves are given in Table 1.

Table 2
Intra-batch and inter-batch accuracy and precision

Level	Concentration added (ng/mL)	Intra-batch				Inter-batch			
		n	Mean concentration found (ng/mL)[a]	RE (%)	%CV	n	Mean concentration found (ng/mL)[b]	RE (%)	%CV
Zopiclone									
LLOQ	0.5	6	0.55	9.1	9.7	18	0.49	−2.7	13.4
LQC	1.5	6	1.42	−5.1	8.4	18	1.43	−4.7	7.1
MQC	50.0	6	51.82	3.6	4.8	18	50.22	0.4	6.0
HQC	137.5	6	126.93	−7.7	7.7	18	131.69	−4.2	3.6
N-desmethyl zopiclone									
LLOQ	0.5	6	0.50	−0.8	6.6	18	0.48	−4.9	14.7
LQC	1.5	6	1.38	−2.8	3.9	18	1.42	−5.6	8.0
MQC	50.0	6	52.00	4.0	3.5	18	49.85	−0.3	6.2
HQC	137.5	6	123.07	−10.5	8.9	18	131.15	−4.6	3.9
Zopiclone-N-oxide									
LLOQ	1.0	6	1.04	3.9	5.6	18	0.96	−3.7	13.4
LQC	3.0	6	2.99	−1.7	6.1	18	2.86	−4.7	9.6
MQC	50.0	6	51.49	3.0	3.0	18	49.61	−0.9	7.0
HQC	137.5	6	127.08	−7.6	8.8	18	128.24	−6.7	5.9

RE: relative error; CV: coefficient of variation; n: total number of observations.
[a] Mean of six replicates at each concentration.
[b] Mean of 18 replicates over three different analytical runs.

Table 3
Stability data for zopiclone (ZOP), N-desmethyl zopiclone (NDZOP) and zopiclone-N-oxide (ZOPNO)

Stability	Compound	Level	Comparison sample[a] concentration (ng/mL) (*A*)	%CV	Stability sample concentration (ng/mL) (*B*)	%CV	%Change, $((B/A) - 1) \times 100$
Bench top (12 h at room temperature)	ZOP	LQC	1.54	9.1	1.36	9.1	−12.7
		HQC	132.67	3.5	129.70	3.8	−2.2
	NDZOP	LQC	1.54	4.9	1.39	3.1	−9.6
		HQC	130.21	5.2	125.28	6.1	−3.8
	ZOPNO	LQC	3.30	6.1	2.91	4.6	−4.0
		HQC	128.16	4.6	124.48	4.7	−2.7
Autosampler (52 h, 10 °C)	ZOP	LQC	1.55	9.1	1.33	4.9	−13.5
		HQC	132.67	3.5	130.08	4.4	−12.0
	NDZOP	LQC	1.54	4.9	1.40	8.5	−9.3
		HQC	130.21	5.2	126.18	7.1	−3.1
	ZOPNO	LQC	3.30	6.1	2.81	8.4	−7.2
		HQC	128.16	4.6	123.23	7.3	−3.8
Freeze–thaw cycle-5	ZOP	LQC	1.45	8.7	1.38	5.3	−4.5
		HQC	133.15	3.2	129.30	4.7	−2.9
	NDZOP	LQC	1.37	9.0	1.37	3.8	−0.6
		HQC	133.08	4.9	128.89	2.9	−3.2
	ZOPNO	LQC	3.01	7.0	2.99	3.5	−0.9
		HQC	127.56	3.7	124.69	6.6	−2.3
Long term (74 days, −20 °C)	ZOP	LQC	1.41	7.0	1.41	3.5	0.2
		HQC	127.37	7.3	140.86	3.2	10.6
	NDZOP	LQC	1.42	12.2	1.59	5.8	12.4
		HQC	128.15	8.6	123.54	1.1	−3.6
	ZOPNO	LQC	2.78	5.1	2.92	4.7	4.9
		HQC	126.74	7.9	130.95	2.8	3.3
Evaporation stability (1 h)	ZOP	LQC	1.55	9.1	1.35	6.4	−13.0
		HQC	132.67	3.5	131.80	2.2	−0.7
	NDZOP	LQC	1.54	4.9	1.34	6.2	−13.0
		HQC	130.21	5.2	127.13	3.1	−2.4
	ZOPNO	LQC	3.30	6.1	2.88	8.5	−5.2
		HQC	128.16	4.6	125.97	2.3	−1.7

CV: coefficient of variation.
[a] Comparison samples prepared by spiking the blank plasma with freshly prepared working solutions before extraction.

For inter-batch, three runs and for intra-batch, a single run was assayed. Each run contains six replicates at each of the four concentration levels (LLOQ, LQC, MQC and HQC). Intra-batch and inter-batch precisions were less than 15% while the accuracy was within 89.5–109.1% for all the three analytes with respect to their nominal concentration as given in Table 2.

The recovery was calculated by comparing the peak area response of extracted sample to the unextracted sample prepared by spiking the respective working solution into the reconstitution solution. The recovery found at LQC, MQC and HQC level was 102.63, 95.76, 103.65 for ZOP; 90.80, 93.48, 102.77 for NDZOP; and 95.80, 97.57, 102.52 for ZOPNO, respectively. The precision (%CV) of recovery among three QC levels found was 4.3, 6.6 and 3.5 for ZOP, NDZOP and ZOPNO, respectively. The recovery of IS was 99.04% with a %CV of 4.7.

3.4. Stability, matrix effect and dilution integrity

Stock solutions of all three analytes and IS were stable at room temperature for 23 h and at 2–8 °C for 36 days. Zopiclone, N-desmethyl zopiclone and zopiclone-N-oxide in control human plasma at room temperature was stable at least for 12 h and for minimum of five freeze and thaw cycles. Process stability was of 52 h at 10 °C. Spiked plasma samples stored at −20 °C for long-term stability experiment were stable for at least 74 days. The evaporation stability was of 1 h. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 3.

The assessment of matrix effect in bioanalysis is extremely important as, even though it may not actually give a signal, but can certainly alter the response of the analytes. Thus, it was tested at different quality control levels for any likely suppression or enhancement of analyte signals. The area response found in unextracted MQC sample was slightly less compared to area response of pure aqueous MQC sample. Thus, minor ion suppression was observed and was about 6–7% for all the analytes. The %CV for ZOP, NDZOP and ZOPNO at LQC and HQC levels were 4.9 and 1.3; 4.8 and 2.4; and 4.5 and 2.9, respectively, which illustrates consistency in matrix effect. Also, the area response of unextracted MQC with and without IS was almost the same, indicating practically no effect of IS on analyte ionization and hence was found suitable for intended purpose.

The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal values. The

Table 4
Summary of selected analytical methods developed for the determination of zopiclone (ZOP) and its metabolites N-desmethyl zopiclone (NDZOP) and zopiclone-N-oxide (ZOPNO) in biological matrices by LC–MS/MS

S. no.	Extraction procedure (matrix, volume); mean recovery	Elution; mobile phase; injection volume	On-column loading at LLOQ	Analytical run time (min)	LLOQ	Ref. no.
1	LLE with DEE–EA (human plasma, 0.5 mL); (ZOP, 109.8%)	Gradient; 5 mM AF (pH 3.0):ACN; 5 μL	250 pg for ZOP	8.0	5.0 ng/mL for ZOP	[17]
2	LLE with MC:DEE (50:50, v/v) (saliva and cellular debris, 0.5 mL); (ZOP, >80%)	Gradient; ACN and FA; 10 μL	10 pg for ZOP	20	0.1 ng/mL for ZOP	[39]
3	SPE (human serum, 0.5 mL); (ZOP, 90.0%)	Gradient; MeOH in 10 mM AA; 20 μL	1.0 ng for ZOP[a]	31.7[b]	5.0 ng/mL for ZOP[a]	[24]
4	SPE (urine, 2 mL); (ZOP, 90.43%)	Gradient; ACN–AF (pH 3.0); 15 μL	30 pg for ZOP	18	0.05 ng/mL for ZOP	[29]
5	– (plasma, –, urine, 5.0 mL); (–)	Gradient; 10 mM AF, pH 3.5 and ACN; 2 μL	–	15.0	0.025 ng/mL for ZOP; 0.1 ng/mL for NDZOP and ZOPNO	[23]
6	LLE with TFA:MeOH (1:50, v/v) (hair and nails, 50 mg); (–)	Gradient; ACN:AF; 20 μL	0.2 pg for ZOP[a]; 0.6 pg for NDZOP[a]	8.0	0.02 pg/mg for ZOP[a] and 0.06 pg/mg for NDZOP[a]	[37]
7	SPE (human plasma, 0.5 mL); (ZOP, 100.68%; NDZOP, 95.68%; ZOPNO, 98.63%)	Isocratic; 0.5% FA in H_2O:ACN:MeOH (25:65:10, v/v/v); 5 μL	4.2 pg for ZOP and NDZOP; 8.4 pg for ZOPNO	4.5	0.5 ng/mL for ZOP and NDZOP; 1.0 ng/mL for ZOPNO	Present method

LLE: liquid–liquid extraction; SPE: solid phase extraction; DEE: diethyl ether; EA: ethyl acetate; MC: methylene chloride; TFA: trifluoroacetic acid; ACN: acetonitrile; AF: ammonium formate; MeOH: methanol; FA: formic acid; AA: ammonium acetate; ATF: ammonium trifluoroacetate.

[a] At LOD.

[b] Retention time.

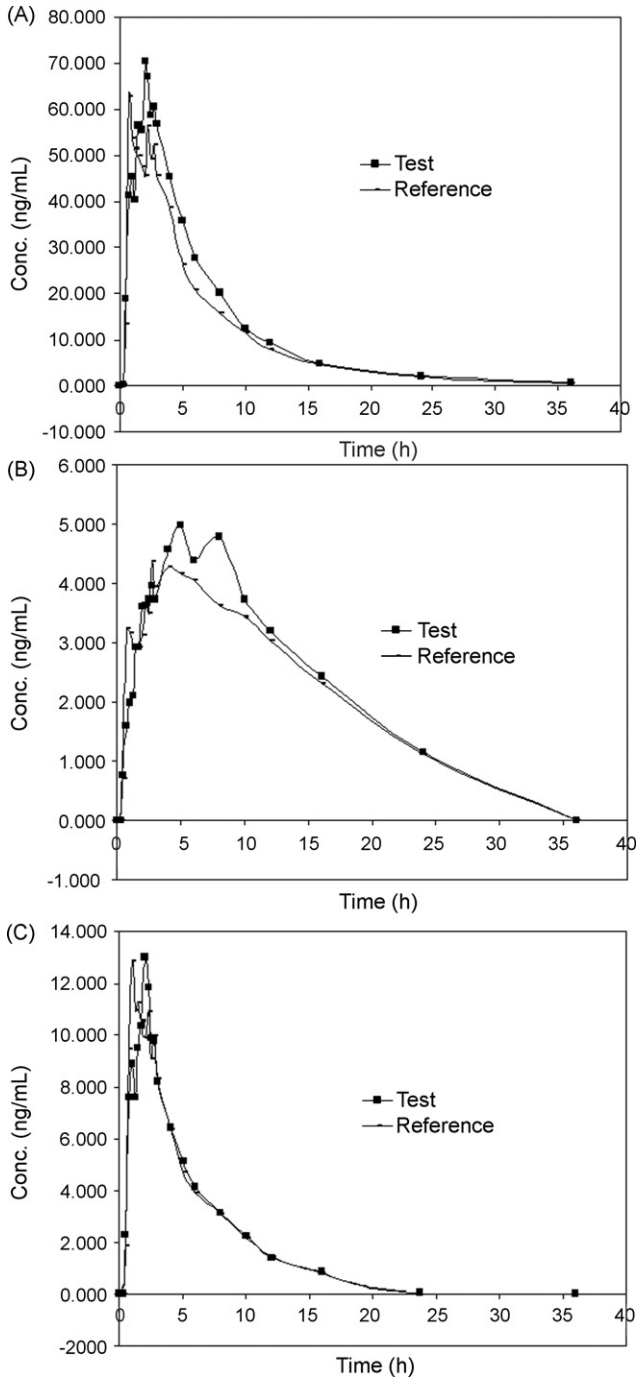

Fig. 7. Mean pharmacokinetic profile of (A) zopiclone, (B) N-desmethyl zopiclone and (C) zopiclone-N-oxide after oral administration of 7.5 mg zopiclone tablet to 16 healthy subjects.

coefficient of variation (%CV) for 1/2 and 1/4 dilution samples of analytes was less than 7.8%.

3.5. Application to analysis of incurred samples

The proposed method was successfully applied to subject samples analysis for a comparative bioavailability study of zopiclone and its metabolites. The reference and test formulations of zopiclone tablet (7.5 mg) were given to 16 healthy human male subjects under fasted condition. All 896 samples including the calibration, QC and subject samples were run and analysed in only 9 days. Not a single batch failed in the entire analysis and all CS and QC samples met the acceptance criteria. Mean plasma concentration versus time profiles for the treatment, under condition of fasting for ZOP, NDZOP and ZOPNO are presented in Fig. 7.

4. Conclusion

The LC–ESI-MS/MS method described for the simultaneous determination of zopiclone and its metabolites, N-desmethyl zopiclone and zopiclone-N-oxide in human plasma is selective, sensitive and rapid. The validated method presents a simple and efficient extraction procedure with reproducible recoveries for zopiclone and its metabolites. There was no interference from endogenous plasma components or other sources and no "cross talk" effect was observed in plasma samples. The validation data demonstrates good precision and accuracy of the method. The established LLOQ is sufficiently low to conduct a pharmacokinetic study of zopiclone and its metabolites with 7.5 mg formulation of zopiclone. A summary of the salient features of LC–MS/MS methods reported for the determination of ZOP, NDZOP and ZOPNO in biological matrices is presented in Table 4.

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