LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY METHOD FOR QUANTIFICATION OF ZOLPIDEM IN HUMAN PLASMA: APPLICATION TO PHARMACOKINETIC STUDY **

Sireesha Dodda [1,2,*], Ajitha Makula [2], Srinivasa Rao Polagani [3], Raj Narayana Kandhagatla [4]

[1] Department of Pharmacy, Anurag group of Institutions (formerly Lalitha College of Pharmacy), Hyderabad, 500088, India: e-mail: rishikavempati@gmail.com
[2] Department of pharmaceutical Sciences, IST, Jawaharlal Nehru Technological University, Hyderabad, 500085, India
[3] AnaCipher Clinical Research Organisation, Hyderabad, 500013, India
[4] Biotechnica Pharma, Hyderabad, 500085, India

A simple, rapid, and sensitive LC-MS/MS method for assay of Zolpidem in human plasma has been developed and validated using Zolpidem D6 as internal standard (IS). The extraction of analyte and IS was done using 100 µL of plasma sample by solid phase extraction with Strata X™ 33 µm polymeric sorbent cartridges. The processed plasma samples were separated using mixture of methanol and 5 mM ammonium acetate buffer in 0.1% formic acid (80:20, v/v) as mobile phase on a C_{18} column at a flow rate of 0.7 mL/min with total run time of 2.0 min. The quantification of the separated components was done in positive ion mode by Multiple Reaction Monitoring (MRM) with mass transitions from m/z 308.0 (parent ion) to m/z 235.0 (product ion) for Zolpidem and from m/z 314.2 (parent ion) to m/z 235.0 (product ion) for the IS. The method was linear in the concentration range of 2.0–200 ng/mL. The recovery was 82.49% and 84.24% for Zolpidem and IS. The inter- and intra- day accuracy and precision were in the range of 94.44 to 103.80% and 2.06 to 8.95%, respectively. The proposed method was successfully applied for pharmacokinetic study of Zolpidem in human volunteers after single oral dose of 10 mg under fasting conditions and incurred sample reanalysis was also performed.

Keywords: *Zolpidem, Zolpidem D6, LC-MS/MS, bioanalytical method development, pharmacokinetic study, incurred sample reanalysis.*

ИСПОЛЬЗОВАНИЕ ЖИДКОСТНОЙ ХРОМАТОГРАФИИ И МАСС-СПЕКТРОМЕТРИИ ДЛЯ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ ЗОЛПИДЕМА В ПЛАЗМЕ КРОВИ ЧЕЛОВЕКА В ИЗУЧЕНИИ ФАРМАКОКИНЕТИКИ

S. Dodda [1,2*], A. Makula [2], S. R. Polagani [3], R. N. Kandhagatla [4]

УДК 543.42.062;543.544

[1] Группа учреждений Анураг (ранее Фармацевтический колледж Лалиты), Хайдарабад, 500088, Индия; e-mail: rishikavempati@gmail.com
[2] Технологический университет Джавахарлала Неру, Хайдарабад, 500085, Индия
[3] Организация клинических исследований AnaCipher, Хайдарабад, 500013, Индия
[4] Biotechnica Pharma, Хайдарабад, 500085, Индия

(Поступила 18 июля 2019)

Разработан простой, быстрый и чувствительный метод жидкостной хроматографии в сочетании с масс-спектрометрией для анализа золпидема в плазме человека с использованием золпидема D6 в качестве внутреннего стандарта (IS). Экстракцию аналита и IS проводили с использованием 100 мкл образца плазмы твердофазной экстракцией с применением картриджей полимерного сорбента Strata X™ 33 мкм. Обработанные образцы плазмы разделяли с помощью смеси метанола и

** Full text is published in JAS V. 87, No. 5 (http://springer.com/journal/10812) and in electronic version of ZhPS V. 87, No. 5 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

5 мкм буфера ацетата аммония в 0/1% муравьиной кислоте (80:20) в качестве подвижной фазы на колонке C_{18} при скорости потока 0.7 мл/мин и общем времени работы 2.0 мин. Количественное определение разделенных компонентов проводили в режиме положительных ионов с помощью мониторинга множественных реакций (MRM) с массовыми переходами от m/z 308.0 (исходный ион) до m/z 235.0 (ион продукта) для золпидема и от m/z 314.2 (исходный ион) до m/z 235.0 (ион продукта) для IS. Метод линейный в диапазоне концентраций 2.0–200 нг/мл. Процент извлечения 82.49% для золпидема и 84.24% для IS. Точность и прецизионность 94.44—103.80 и 2.06—8.95% соответственно. Метод успешно применен для фармакокинетического исследования золпидема на добровольцах после однократной пероральной дозы 10 мг, а также проведен повторный анализ взятых образцов.

Ключевые слова: *золпидем, золпидем D6, LC-MS/MS, разработка биоаналитических методов, фармакокинетическое исследование, повторный анализ взятых образцов.*

Introduction. Zolpidem, chemically N,N dimethyl-2-[6-methyl-2-(4-methylphenyl)imidazo[1,2-a]pyridin-3-yl]acetamide is a hypnotic drug used to improve sleep in insomnia patients [1]. Though not effective in maintaining the sleep, it initiates sleep [2]. It is a non-benzodiazepine hypnotic (imidazopyridine derivative) and binds to gamma aminobutyric acid $GABA_A$ receptors at the same location as benzodiazepines [3]. It selectively binds to $GABA_A$ receptors and thus has weak anticonvulsant and anxiolytic properties but very strong hypnotic properties [4].

As per the available literature, there were few LC-MS/MS based methods proposed for analysis of Zolpidem alone [5–8] or along with its metabolites [9, 10] or in combination with other drugs in biological fluids [11–15]. The proposed methods for simultaneous analysis of Zolpidem with its metabolites or with other drugs used longer run times, which are not suitable for bioavalability/bioequivalence (BA/BE) studies and therapeutic drug monitoring (TDM) of Zolpidem alone. Kintz P et al. [5] proposed a LC-MS/MS method for determination of Zolpidem in oral fluid using Diazepam D5 as the internal standard with a total chromatographic run time of 6.0 min. The method proposed by Jignesh B et al. [6] used Es-citalopram as internal standard and 200 µL of human plasma with run time of 3.2 min. Both the methods proposed by Reddy DC et al. [7] and Kuldeep KN et al. [8] used Mirtazapine as internal standard, employing more than 200 µL of human plasma with run time ≥2.5 min.

The present work describes simple, robust, and rapid LC-MS/MS assay method for quantification of Zolpidem in human plasma. The method gives improved reliability by the usage of deuterated analogue Zolpidem D6 as the IS. The deuterated analogues as IS have the same extraction recovery, ionization response in electro spray (ESI) mass spectrometry, and the same chromatographic retention time [16, 17]. Hence, the chromatography time is reduced and the assay method becomes more robust with high throughput and lower rejection rates [18]. The use of biological fluid is also minimized by employing only 100 µL human plasma for sample processing with single step solid phase extraction (SPE) technique giving cleanest samples. The short chromatographic run time of 2.0 min makes it possible to analyze more than 300 samples per day.

Materials and methods. *Standards and chemicals.* The working standards of Zolpidem (99.74%) and Zolpidem D6 (98.01%) were procured from Clearsynth Labs Ltd. (Mumbai, India). HPLC grade water was purchased from Rankem (Gurugram, India), and HPLC grade methanol was obtained from J.T Baker (Phillipsburg, USA). Analytical grade reagents of formic acid and ammonium acetate were procured from Merck Ltd. (Mumbai, India). K_2-EDTA control human plasma was obtained from Deccan's pathological Labs (Hyderabad, India). Strata X™ 33 µm polymeric sorbent cartridges (30 mg, 1 cm^3) were purchased from Phenomenex (CA, USA).

LC-MS/MS instrument and conditions. An HPLC system (Shimadzu, Kyoto, Japan) equipped with Discovery® HS C18 (50×4.6 mm, 5 µm) and a binary LC-20AD pump was used for this work. A mobile phase composed of mixture of methanol and 5 mM ammonium acetate buffer in 0.1% formic acid (80:20, v/v) at flow rate of 0.7 mL/min was used for separation of Zolpidem from IS and endogenous matrix components. Quantification of the separated components was done with MS-MS detection using AB Sciex API 2000 mass spectrometer (Foster city, CA, USA) in positive ion mode, equipped with Turboion spray™ interface at 550°C.

The ion spray voltage set was 5000 V. The source dependent parameters, viz. nebulizer gas, auxiliary gas, curtain gas, and collision gas were set at 40, 30, 35, and 6 psi, respectively. The compound dependent parameters set were declustering potential, collision energy, entrance potential, and collision cell exit potential at 30, 44, 10, and 8 V, respectively both for Zolpidem and IS. MRM mode was used for detection of ions, and the transitions were carried out for Zolpidem from *m/z* 308.0 (parent ion) to *m/z* 235.0 (product ion) and for IS from *m/z* 314.2 (parent ion) to *m/z* 235.0 (product ion). Unit resolution was set for Q1 and Q3

quadrupoles. Dwell time of 200 ms was set. The data analysis was performed by Analyst software™ (version 1.4.2).

Preparation of calibration curve standards and quality control samples. Two primary stock solutions of Zolpidem were prepared at concentration of 1 mg/mL in HPLC grade methanol. A stock solution of IS was also prepared at same concentration in same solvent. The working solutions of Zolpidem were prepared at appropriate concentrations using diluent (methanol:water; 60:40, v/v). The working solution of IS was also prepared in the same diluent at a concentration of 1000 ng/mL.

To prepare calibration curve (CC) standards 4.75 mL of K_2-EDTA human plasma were spiked with 0.25 mL of appropriate working solutions of Zolpidem. CC standards were prepared at nine non-zero concentrations of 2.028, 4.056, 10.141, 20.282, 40.564, 81.129, 121.088, 161.450, and 201.813 ng/mL. Similarly, quality control (QC) samples were prepared at concentrations of 2.031 (Lower Limit of Quantitation Quality Control, LLOQ QC), 5.317 (Lower Quality Control, LQC), 25.319 (Middle Quality Control, MQC-1), 101.277 (MQC-2), and 153.450 ng/mL (High Quality Control, HQC).

Sample preparation protocol. An aliquot of 100 µL of human plasma was mixed with 10 µL of IS working solution and 500 µL water. The solid phase extraction (SPE) cartridges (Strata X™ 33 µm, polymeric sorbent) were pre-conditioned with 1 mL methanol, followed by 1 mL water. Then the plasma sample was loaded onto cartridges and washed with 1 mL of 50 mM ammonium acetate buffer followed by 2 mL water (1 mL each time). Elution was carried out with 0.6 mL of mobile phase and 15 µL was injected into LC-MS/MS system.

Method validation. A complete validation of the developed method was carried out as per USFDA guidelines [19]. The parameters evaluated were selectivity, sensitivity, matrix effect, precision and accuracy, dilution integrity, recovery, stability, ruggedness, and run size evaluation.

Pharmacokinetic study design. The developed method was used for pharmacokinetic (PK) study of Zolpidem 10 mg in six healthy South Indian male subjects under fasting conditions. The study protocol was approved by Maarg Independent Ethics Committee, Hyderabad and the volunteers provided with written consent. The blood samples were collected at pre-dose and 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 3.0, 4.0, 5.0, 6.0, 8.0, and 12.0 h after oral administration of 10 mg Zolpidem tablet into K_2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The plasma was separated by centrifugation at 4000 rpm and 4°C for 10 min and was stored at -70±10°C until use. WinNonlin software (version 5.2) was used for calculating PK parameters of Zolpidem. An incurred sample reanalysis (ISR) was also performed by choosing two samples from each subject, one near C_{max} and the other at elimination phase of mean plasma concentration – time profile of Zolpidem.

Results and discussion. *Mass spectrometry.* A tuning solution of 500 ng/mL of Zolpidem and IS was injected into ESI source and tuned in both positive and negative ionization modes. The intense signal was produced by positive mode and hence selected for study. Data acquisition was done in MRM mode for better selectivity of the analyte from endogenous matrix components. The source dependent and compound dependent parameters were suitability altered to get reproducible response and intense signal for both Zolpidem and IS. The most sensitive transition was finally observed from *m/z* 308.0 (parent ion) to *m/z* 235.0 (product ion) for Zolpidem and from *m/z* 314.2 (parent ion) to *m/z* 235.0 (product ion) for IS (Fig. 1).

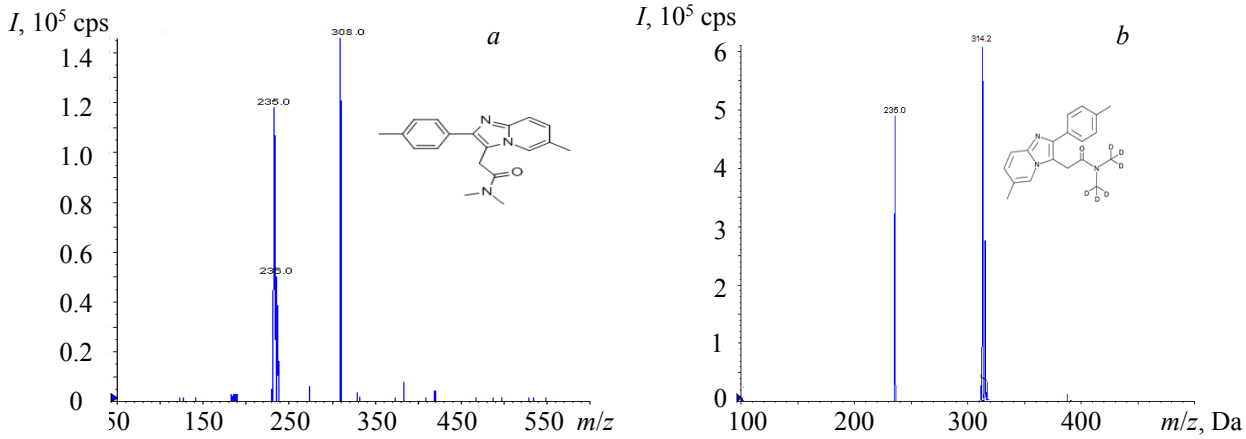

Fig. 1. Product ion mass spectra of Zolpidem (a) and Zolpidem D6 (b).

Optimization of chromatographic conditions. Good sensitivity and selectivity of the assay was obtained by proper optimization of mobile phase composition, flow rate, and analytical column. Different buffers like ammonium formate and ammonium acetate of different strength were verified in combination with organic solvents like acetonitrile and methanol in isocratic mode for the suitability as mobile phase. Finally, a mixture of methanol and 5 mM ammonium acetate buffer in 0.1% formic acid (80:20, v/v) as mobile phase and Discovery® HS C18 (50×4.6 mm, 5 µm) column gave satisfactory peak shape and response even at smallest concentration level (LLOQ QC) of the Zolpidem and IS. The total run time set was 2.0 min with a retention time of 0.85±0.5 min for both Zolpidem and IS at mobile phase flow rate of 0.7 mL/min.

Optimization of sample preparation method. Maximum recovery with no or minimal matrix effect is obtained with appropriate extraction technique. Protein precipitation was performed with precipitating agents like ethanol, methanol, and acetonitrile, but the % recovery obtained was poor. The liquid-liquid extraction technique with dichloromethane, diethyl ether, and ethyl acetate was tried, and the recovery was inconsistent. Hence, SPE technique using Strata X™ 33 µm, polymeric sorbent cartridges was tried using mobile phase itself for elution of analyte and IS, and satisfactory results in terms of recovery and matrix effect were obtained.

Chromatography and selectivity. Selectivity of the method was evaluated by analysis of eight lots of blank plasma derived from different sources. There was no direct interference observed from endogenous components in blank plasma at the retention times of Zolpidem and IS, as shown in Fig. 2a, and also there was no significant interference from the IS on the MRM channel of Zolpidem (Fig. 2b).

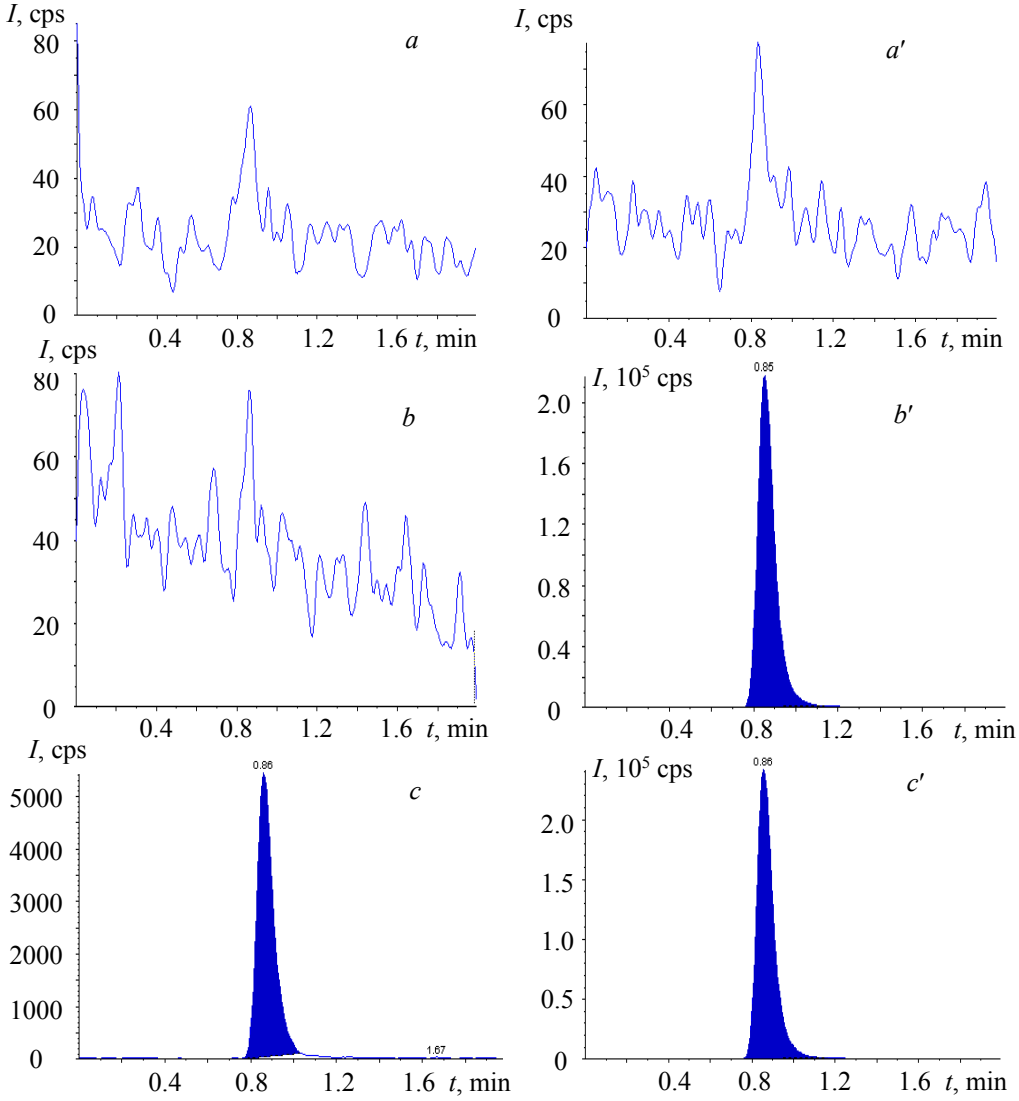

Fig. 2. Typical MRM chromatograms of Zolpidem (a–c) and IS (a′–c′) in (a, a′) double blank plasma (without Zolpidem and IS), (b, b′) blank plasma with IS, and (c, c′) LLOQ QC sample.

Sensitivity. The LLOQ QC was set at 2.0 ng/mL for Zolpidem and the signal to noise ratio (*S*/*N*) was measured. The method proved to be sensitive with $S/N \geq 10$. The accuracy and precision (%CV) at this concentration was found to be 100.60% and 1.58%, respectively (Fig. 2c).

Matrix effect. Effect of matrix ions was evaluated using total eight lots of human plasma, including one hemolytic and one lipemic plasma. The mean IS normalized factors obtained were 0.977 and 0.990, respectively at LQC and HQC levels of Zolpidem.

Linearity, precision and accuracy. The linearity of total five calibration curves in the concentration range of 2.0 to 200 ng/mL was satisfactory with correlation coefficient $r^2 > 0.99$. The accuracy and precision (%CV) for CC standards ranged from 98.88 to 101.59% and 0.67 to 2.28%, respectively.

The precision (%CV) and accuracy was assessed at both intra-day and inter-day by running five batches of QC sample (Table 1). The intra-day accuracy ranged from 94.44 to 98.38%, and precision (%CV) ranged from 2.06 to 4.67%. Similarly, inter-day accuracy was in the range of 100.06 to 103.80% and precision (%CV) was in the range of 4.08 to 8.95%.

TABLE 1. Intra- and inter-day precision and accuracy data

QC	Conc. Spiked, ng/mL	Intra-day precision and accuracy (n = 12; 6 from each batch)			Inter-day precision and accuracy (n = 30; 6 from each batch)		
		Conc. Found (mean; ng/mL)	Precision, %CV	Accuracy, %	Conc. Found (mean; ng/mL)	Precision, %CV	Accuracy, %
LLOQ	2.031	1.918	4.67	94.44	2.108	8.95	103.80
LQC	5.317	5.096	3.68	95.85	5.320	5.17	100.06
MQC-1	25.319	24.790	2.06	97.91	25.733	4.08	101.64
MQC-2	101.277	99.640	2.27	98.38	103.999	4.44	102.69
HQC	153.450	150.367	2.69	97.99	156.321	4.25	101.87

Recovery and dilution integrity. The % recovery obtained with proposed SPE procedure was good and reproducible for both Zolpidem and IS. The mean % recovery of Zolpidem was 82.49%, with precision (%CV) in the range of 0.61 to 2.21%, and the % recovery of IS was 84.24%.

The concentration of real time plasma samples exceeding the ULOQ concentration can be quantified by dilution of the sample with blank matrix. The dilution integrity (DI) was assessed by extending the ULOQ concentration to 674.170 ng/mL using two- and four-times dilution with human blank plasma. The precision (%CV) for DI of two- and four-fold dilution was found to be 0.70% and 0.81%, respectively, and accuracy was found to be 101.88% and 99.31%, respectively.

Stability studies. The stability of analyte in plasma samples under various conditions like bench top stability (7 h), freeze and thaw stability (4 FT cycles), short-term stability at –20±5°C for 3 days, and long-term stability at –70±10°C for 77 days were assessed at LQC and HQC levels. In addition, processed sample stability (re-injection stability, 50 h; auto-sampler stability, 33 h; wet extract stability at 2–8°C, 33 h) was also assessed. The results were found to be within the acceptable limits and are summarized in Table 2.

Ruggedness and run size evaluation. One precision and accuracy batch was evaluated using different sets of reagents and columns of the same make but of different batch numbers on different instruments of the same make. The precision (%CV) and accuracy results were in the range of 1.92 to 4.46% and 106.16 to 114.15%, respectively.

Forty sets of QC samples at LQC, MQC-1, MQC-2 and HQC levels were analyzed along with fresh CC standards and QC samples to perform run size evaluation (RSE). All the 160 RSE samples and 24 fresh QC samples of Zolpidem were within 15% of their respective nominal concentrations. The precision (%CV) and accuracy results were in the range of 1.12 to 1.92% and 101.17 to 103.56% for RSE samples and 0.90 to 1.69% and 102.16 to 104.49% for fresh QC samples, respectively.

Pharmacokinetic study and incurred sample reanalysis. The proposed method was applied successfully to quantify Zolpidem in real subject samples during PK study in six healthy human volunteers under fasting conditions (Fig. 3). The PK parameters calculated are summarized in Table 3, and the mean plasma (±SD) concentration versus time profile of Zolpidem is shown in Fig. 4. An ISR was also performed, and the %variability obtained was within ±15% (Table 4), indicating that the proposed method is rugged.

TABLE 2. Stability studies of Zolpidem under different conditions (n = 6)

Stability	Storage condition	Level	Conc. Spiked, ng/mL	Conc. Found (mean; ng/mL)	% Stability	Precision, %CV
Wet extract stability	Room temperature (33 h)	LQC HQC	5.317 153.450	5.625 165.609	*100.07* *101.22*	1.16 2.00
Re-injection stability	50 h at 15°C	LQC HQC	5.317 153.450	5.647 156.366	103.20 98.14	2.34 1.07
Auto-sampler stability	Auto-sampler temperature (15°C, 33 h)	LQC HQC	5.317 153.450	5.799 166.039	103.17 101.48	1.36 1.99
Freeze-Thaw stability	After 4th cycle at –70±10°C	LQC HQC	5.317 153.450	5.646 164.365	100.46 100.46	1.46 1.05
Short term stability	3 days at –20±5°C	LQC HQC	5.317 153.450	5.483 160.540	97.55 98.12	1.83 0.70
Long term stability	77 days at –70±10°C	LQC HQC	5.317 153.450	5.438 164.393	101.32 99.50	1.94 1.72
Bench top stability	Room temperature (7 h)	LQC HQC	5.317 153.450	5.359 159.378	95.35 97.41	2.48 1.48

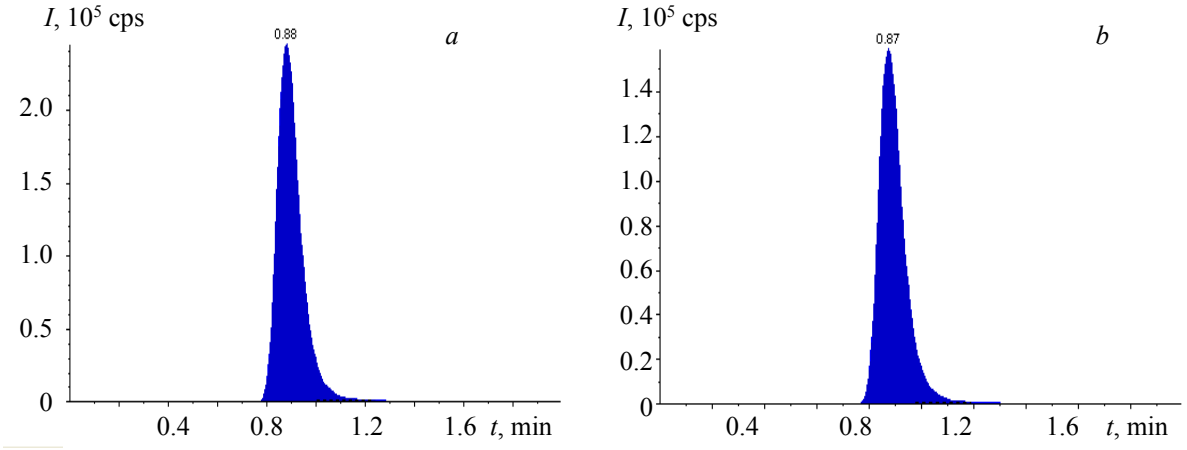

Fig. 3. Typical MRM chromatograms of Zolpidem (a) and IS (b) from the analysis of 1.25 h subject plasma sample after administration of Zolpidem 10 mg.

TABLE 3. Pharmacokinetic parameters of Zolpidem (n = 6, mean ± SD)

Parameter	Mean ± SD
C_{max} (ng/mL)	154.80±14.86
t_{max} (h)	0.54 ± 0.10
$AUC_{0\to t}$ (ng h/mL)	630.97 ± 170.56
$AUC_{0\to\infty}$ (ng h/mL)	709.87 ± 236.98
$t_{1/2}$ (h)	3.90 ± 1.23
Kel (h^{-1})	0.19 ± 0.05

TABLE 4. Incurred Sample Reanalysis data of Zolpidem

Subject No.	Sampling point, h	Initial conc., ng/mL	Re-assay conc., ng/mL	Mean	Difference, * %
1	0.75	151.071	158.576	154.824	4.847
1	8.00	12.968	11.483	12.226	12.147
2	0.75	132.725	136.538	134.632	2.832
2	12.00	14.344	15.952	15.148	10.615
3	0.50	182.219	189.384	185.802	3.856
3	12.00	26.735	23.568	25.152	12.592
4	0.75	121.924	129.975	125.950	6.392
4	12.00	7.941	6.884	7.413	14.260
5	0.75	118.109	120.583	119.346	2.073
5	12.00	6.206	7.106	6.656	13.522
6	0.75	117.439	111.756	114.598	4.959
6	12.00	14.497	16.652	15.575	13.837

* Expressed as [(initial conc. – re-assay conc.)/mean]×100.

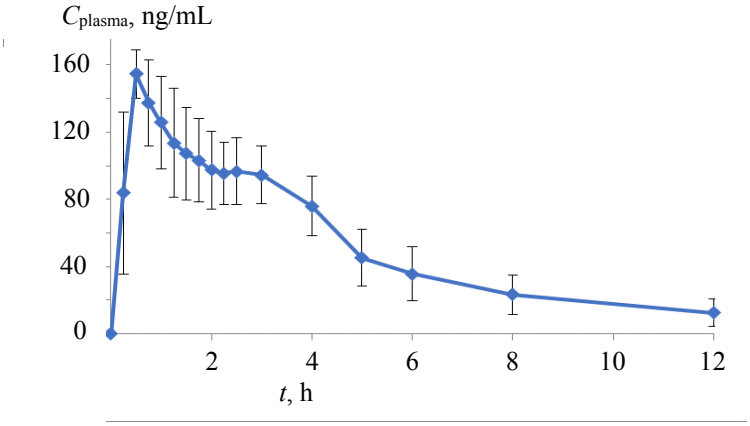

Fig. 4. Mean (±SD) plasma concentration – time profile of Zolpidem after oral administration of Zolpidem 10 mg tablet.

Conclusions. The LC-MS/MS based bioanalysis of Zolpidem in human plasma using the proposed method is rapid, simple, and sensitive enough for PK or BA/BE studies. Consistent and reproducible results, and also little variability in recovery, were obtained by the use of a deuterated analogue of Zolpidem, i.e., Zolpidem D6 as the internal standard. Extraction of the sample employing only 100 µL of plasma with SPE in a single step, avoiding the evaporation and reconstitution steps, gave the clean samples in reduced sample processing time. More than 300 samples can be analyzed per day as the total run time per analysis is 2.0 min. The suitability of the method was successfully verified by PK studies in humans. From the results obtained during validation and PK study, the conclusion can be made that the proposed method can be applied for BA/BE studies and routine TDM of Zolpidem.

Acknowledgments. The authors are grateful to AnaCipher Clinical Research Organisation, Hyderabad, India for providing necessary facilities for carrying out this work.

REFERENCES

1. S. Budavari, *The Merck Index*, 12th ed., White house Station (NJ), Merck and Co Inc. (1996).
2. R. P. Rosenberg, *Ann. Clin. Psychiatry*, **18**, 49–56 (2006).
3. B. Lemmer, *Physiol. Behav.*, **90**, 285–293 (2007).
4. P. Salva, J. Costa, *Clin. Pharmacokinet.*, **29**, 142–153 (1995).
5. P. Kintz, M. Villain, B. Ludes, *J. Chromatogr. B*, **811**, 59–63 (2004).

6. B. Jignesh, A. Jangid, R. Shetty, B. Shah, S. Kambli, G. Subbaiah, S. Singh, *Biomed. Chromatogr.*, **20**, 736-742 (2006).
7. D. C. Reddy, A. T. Bapuji, V. S. Rao, V. Himabindu, S. Ravinder, *J. Chromatogr. Sci.* **50**, 538–546 (2012).
8. K. N. Kuldeep, K. S. Manoj, S. Swapnil, D. Jaya, *Int. J. Chem. Anal. Sci.*, **5**, 99–106 (2014).
9. Y. Shi, P. Xiang, B. Shen, *J. Chromatogr. B*, **911**, 140–146 (2012).
10. Przemysław P, Szymon B, Karol S, B. Bogusław *Adv. Med. Sci.* **60**, 167–172 (2015).
11. M. Laloup, M. Fernandez, D. G. Boeck, M. Wood, V. Maes, M. Samyn, *J. Anal. Toxicol.* **29**, 616–626 (2005).
12. S. J. Marin, M. Roberts, M. Wood, G. A. McMillin, *J. Anal. Toxicol.* 6: 472–476 (2012).
13. D. Remane, M. R. Meyer, D. K. Wissenbach, H. H. Maurer, *Anal. Bioanal. Chem.*, **401**, 1341–1352 (2011).
14. I. Tomomi, K. Keiko, H. Makiko, I. Noriaki, *J. Chromatogr. B*, **877**, 2652–2657 (2009).
15. J. Moonhee, C. Hyejin, Y. Wonkyung, C. Hyeyoung, K. Eunmi, Y. Bum-Hee, O. Yunhye, C. Heesun, *J. Pharm. Biomed. Anal.*, **74**, 213–222 (2013).
16. http://www.aptochem.com/t-bioanalysis.aspx
17. E. Stokvis, H. Rosing, L. López-Lázaro, J. H. Schellens, J. H. Beijnen, *Biomed. Chromatogr.*, **18**, 400–402 (2004).
18. R. L. Wolen, *J. Clin. Pharmacol.*, **26**, 419-424 (1986).
19. US DHHS, FDA and CDER. (2001). Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine. Available at: http://www/ fda.gov/cder/guidance/index.htm.