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RESEARCH ARTICLE

Bioanalytical HPTLC Method for Estimation of Zolpidem Tartrate from Human Plasma

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ABSTRACT

A simple and selective high performance thin layer chromatographic (HPTLC) method was developed and validated for the estimation of zolpidem tartrate from human plasma using eperisone hydrochloride as an internal standard (IS). Analyte and IS were extracted from human plasma by liquid liquid extraction (LLE) technique. The Camag HPTLC system, employed with software winCATS (ver.1.4.1.8) was used for the proposed bioanalytical work. Planar chromatographic development was carried out with the help of Silica Gel 60 F_{254} precoated TLC plates. The optimized mobile phase was composed of ethyl acetate: methanol: acetonitrile (7:1.5:1.5, v/v/v).The detection of spots was carried out densitometrically using a UV detector at 298nm in absorbance mode. In HPTLC densitogram well defined peak was obtained for zolpidem tartrate with peak start position at 55 hR_f, max position at 59 hR_f and end position at 63 hR_f. The optimal hR_f value for zolpidem tartrate and IS were found to be 58 and 20 hR_f respectively. Performance characteristics of HPTLC method for estimation of zolpidem tartrate from human plasma were statistically validated as per the validation protocol designed based on the recommendations given by European Medicines Agency (EMA) guidelines of bioanalytical method validation. With validated method regression analysis of the calibration data showed a good linear relationship with mean correlation coefficient (r^2) 0.998. The method was found to be simple and selective. The proposed method can be effectively used for accurate and precise determination of zolpidem tartrate from human plasma and can be applied for therapeutic drug monitoring and pharmacokinetic (Pk) studies on real clinical samples.

KEYWORDS

HPTLC, Zolpidem Tartrate, Eperisone Hydrochloride, EMA, Bioanalytical Method Validation

INTRODUCTION

Bioanalysis deals with analysis of drugs, their metabolites and/or endogenous substances in the biological matrices such as plasma, urine, serum, blood and tissue extracts. A properly validated bioanalytical method for quantitative determination of drugs and their metabolites plays an important role in the effective performance of bioequivalence, pharmacokinetic and toxicokinetic studies[1,2].

Zolpidem tartrate, chemically known as N, N, 6-Trimethyl-2-ptolyl-imidazo (1,2-0a) pyridine-3-acetamide L-(+)-tartrate is a non-benzodiazepine hypnotic agent binds preferentially to one benzodiazepine receptor subtype ω-1 benzodiazepine-1[3]. Zolpidem tartrate acts as a sleep inducer without anti-convulsant and muscle relaxant effects[3,4]. The hypnotic effect of zolpidem tartrate is same as that of drugs which are comes under benzodiazepine class, but it's

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structurally different from benzodiazepine and classified as imidazopyridine[5].

The reported analytical methods for determination of zolpidem tartrate from human plasma includes High performance liquid chromatography with ultraviolet detection[6,7], photodiode array detection[8] and tandem mass spectrometry[5,9] detection, nitrogen phosphorous[10,11] detection and Gas chromatography with mass spectrometry[12,13] detection. However, most of these methods were found to be time consuming and expensive in terms of sample preparation technique and analysis run time. The referred scientific literature indicated that no HPTLC method is available for estimation of zolpidem tartrate from human plasma. As HPTLC is a sophisticated technique which provides good separation of analyte from interfering components along with its qualitative and quantitative analysis in shorter time with low consumption of mobile phase. The HPTLC technique is economical as compared to other techniques. On the basis of literature, scientific rationale and need of research work, method development was carried out to attain the objectives of developing a new, simple, selective, rapid and cost-effective bioanalytical method for estimation of zolpidem tartrate using HPTLC.

MATERIAL AND METHODS

Chemical and Reagents

Zolpidem tartrate (Figure 1a) and Eperisone hydrochloride(IS) (Figure 1b) were obtained for the purpose of academic research from local pharmaceutical company. All chemicals were purchased from S.D. Fine Chemicals, Mumbai, India. HPTLC Aluminium plates precoated with Silica Gel 60 F_{254} were purchased from Merck (20×20 cm). Human pooled plasma was obtained from Sir J.J. Mahanagar Raktapedhi, Mumbai, and stored in deep freezer at -20° C until used.

Instruments

HPTLC analysis was done using CAMAG Linomat 5 applicator equipped with TLC Scanner 3. Bandwise applications were done by using Hamilton syringe, capacity of 100μL. Instrument operation, data collection and integration were accomplished using winCATS software version 1.4.1.8.

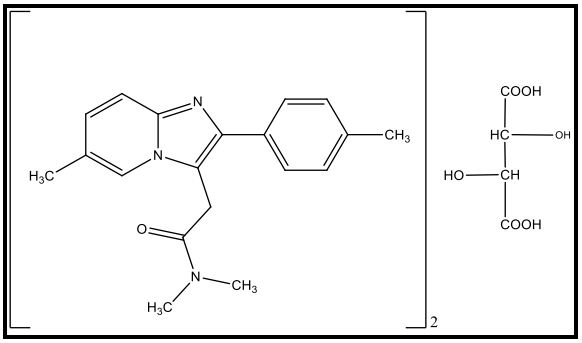

Figure 1a: Chemical structure of zolpidem tartrate

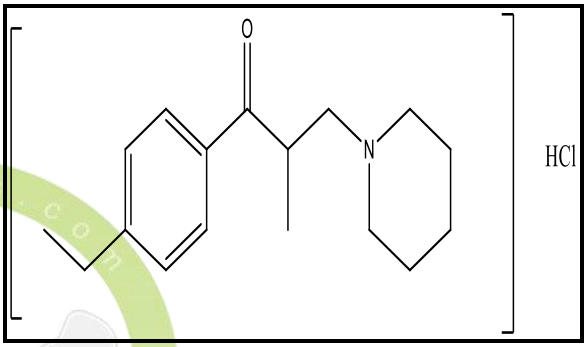

Figure 1b: Chemical structure of eperisone hydrochloride

Preparation of Stock Solutions and Working Solutions

Standard stock solution of zolpidem tartrate and IS were prepared by dissolving 10mg of drug in 100mL methanol to obtain concentration 100μg/mL (100ppm) respectively. The standard stock solution of 100μg/mL zolpidem tartrate was further diluted with methanol to obtain various working standard solutions with concentrations 0.1, 0.5, 0.9, 1.3, 1.7, 2.1 and 2.5μg/mL. The IS working solution of 90μg/mL was prepared by diluting stock solution with methanol. All the solutions were stored between 2°C - 8°C until use.

Preparation of Calibration Standards and Quality Control Samples

Calibration standards were prepared by spiking 360μL of blank plasma with 40μL of respective working standard solution of zolpidem tartrate to get concentrations of 100, 500, 900, 1300, 1700, 2100 and 2500 ng/mL. Quality Control (QC)

samples were prepared by spiking plasma with working standard of drug to obtain concentrations of 500ng/mL (Low QC), 1300ng/mL (Mid QC) and 2100ng/mL (High QC).

The proposed research work was carried out and it is presented in following two sections 3 and 4.

BIOANALYTICAL METHOD DEVELOPMENT (BMD)

BMD –Experimental

Sample Preparation

Biological samples are extremely complex matrices comprised of many components that can interfere with good separation hence; sample preparation is an important aspect of bioanalytical method development. Sample preparation was done to extract the analyte of interest from spiked plasma using LLE technique. Various extracting solvents were tried for LLE such as ethyl acetate, dichloromethane, diethyl ether and *tert*-butyl methyl ether (TBME). With each solvent sample was prepared as follows.

In eppendrof tube 360μL of plasma, 40μL of drug working standard solution and 40μL of IS (90μg/mL) were added. After mixing it by hand shaking, 1.6mL of extracting solvent was added with the help of micropippette. The eppendrof tubes were placed on a vortex mixer and vortexed for 2 minutes. Then the tubes were centrifuged at 5500 rpm for 15 minutes at -15° C in cold centrifuge. After 15 minutes of centrifugation the supernatant clear aliquot (about 1.3mL) from the centrifuge tube was transferred into a separate glass tube (5mL). An aliquot was then evaporated to dryness under the stream of nitrogen (1 bar) by using nitrogen evaporator (MiniVap). The dry residue obtained after evaporation was reconstituted with 100μL of methanol. This reconstituted sample solution was applied on the HPTLC plate.

Sample Application

Bandwise reconstituted samples were applied on Silica Gel 60 F_{254} precoated TLC plates (10 cm x 10 cm) using HPTLC Linomat 5 sample applicator with the help of 100μL Hamilton syringe. Band length was kept as 6mm at a constant flow rate of 0.15μL/sec by using a nitrogen aspirator.

Chromatography

10mL of optimized mobile phase composed of ethyl acetate: methanol: acetonitrile (7:1.5:1.5 v/v/v) was poured from the edge of whatman filter paper which was exactly adjusted to the side glass wall of Camag twin-trough chamber (10×10cm) to avoid a smiley-effect of the front. Chamber was saturated for 30 min, after chamber saturation applied plates were subjected for ascending development with migration distance of 80mm (Measured from bottom plate edge).

Densitometry

Developed plates were air dried and scanned densitometrically using a UV detector at 298 nm in absorbance mode with slit dimensions of5×0.30mm.

BMD- Results and Discussions

Sample Preparation and its Optimization

Sample preparation for was performed using LLE technique and optimized. Amongst the number of selected and screened solvents, diethyl ether gave clear supernatant aliquot with good recovery. The chromatogram of blank plasma sample showed no interferences at hR_f of the zolpidem tartrate and IS, making the method selective. Parameters for sample preparation experiments namely, volume of diethyl ether added, vortexing time, centrifugation temperature and time, evaporation temperature, evaporation time were optimized. All optimized sample preparation parameters are tabulated in table 1.

Table 1: Optimized parameters for sample preparation

1.	Volume of diethyl ether	1.6mL
2.	Vortexing time	2 minutes
3.	Centrifugation temperature and time	-15° C, 15 minutes
4.	Evaporation temperature	60° C
5.	Evaporation time	5 minutes

Chromatographic Conditions

Bioanalytical method of zolpidem tartrate and eperisone hydrochloride (IS) was developed using trial and error approach. All chromatographic conditions are tabulated in table 2.

Table 2: Optimized chromatographic conditions

1.	Mobile phase composition	Ethyl acetate: methanol: acetonitrile (7:1.5:1.5, v/v/v)
2.	Application volume	70μL
3.	Band length of spots	6 mm
4.	Chamber saturation time	30 minutes
5.	Migration distance	80mm
6.	Detection wavelength	298 nm

Densitogram obtained using optimized chromatographic conditions for zolpidem tartrate and IS is shown in figure 2, hR_f value for zolpidem tartrate and IS were found to be 58 and 20 respectively.

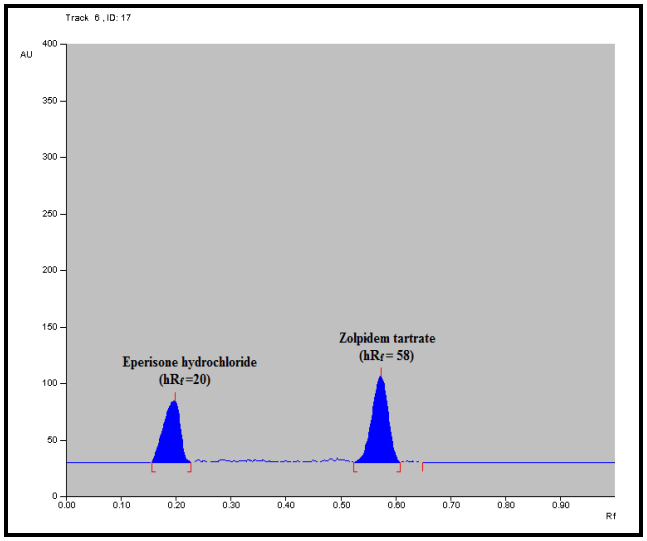

Figure 2: Densitogram of zolpidem tartrate with eperisone hydrochloride (IS)

BIOANALYTICAL METHOD VALIDATION (BMV)

BMV- Experimental

The developed bioanalytical HPTLC method was validated as per the validation protocol (table 3) designed on the basis of recommendations given by EMA guidelines for validation of bioanalytical procedures[14].

BMV- Results and Discussions

Selectivity

Densitograms of blank plasma and LLOQ sample of zolpidem tartrate are shown in figure 3a and 3b respectively. There were no interfering peaks observed at hR_f values of zolpidem tartrate and IS. The results indicated that method exhibits good selectivity.

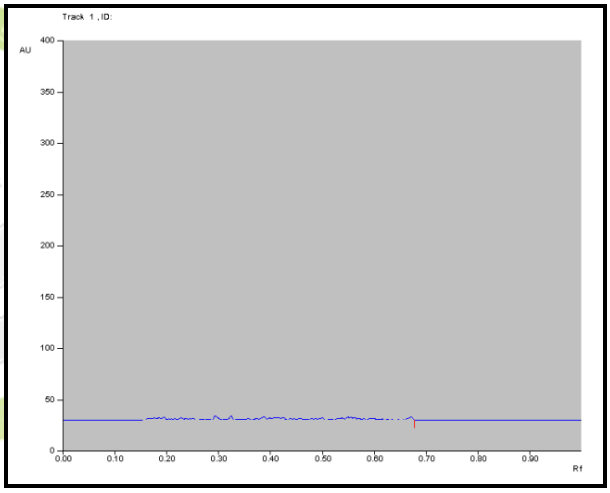

Figure 3a: Densitogram of blank human plasma

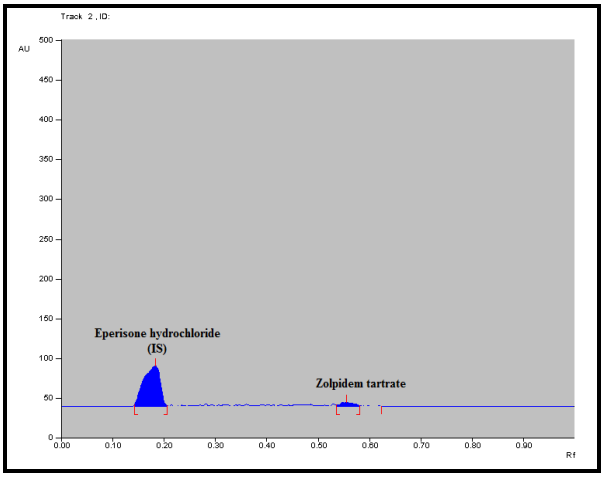

Figure 3b: Densitogram of LLOQ of zolpidem tartrate

Table 3: Validation protocol

Sr. No.	Parameters	Experiment	Evaluation for	Acceptance criteria
1.	Selectivity	Analyzed sources of blank plasma samples	Interference at the retention time of the analyte and IS	No interfering signals should be present. If present, should be less than 20% of the response signal for lower limit of quantificaton (LLOQ) and 5% of the response signal for I.S. at their respective hR_f values
2.	Carry-over	The extracted blank sample was applied immediately after the application of upper limit of quantification (ULOQ) standard sample	To check any interfering peak at the hR_f values of drug and internal standard	**For drug** Not more than 20% of the LLOQ response **For IS** not more than 5% of the response
3.	Lower limit of quantificaton (LLOQ)	Applied 5 replicates of lowest calibration standard	Detection of LLOQ	Analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample
4.	Calibration curve	Analyzed of 6 concentration levels with a blank and zero sample	Analysis of the behavior of variance across the validation range	Back calculated values should be within 15% of the nominal value, except for LLOQ for which it should be within 20%, at least 75% of the calibration standard should be fit in the criteria
5.	Accuracy and precision	Five replicates of QC samples at four concentration levels; LLOQ, low QC (LQC), mid QC (MQC) and high QC (HQC) were analyzed on four different days to determine within-run and between-run accuracy and precision. Each run consisted of one blank sample, one zero sample, one standard curve containing all calibration standards along with five replicates each of QC samples and LLOQ.	For percent nominal values and coefficient of variation	Percent relative error (%RE) should be within ±15% (±20% for LLOQ) of the nominal value and the percent coefficient of variation (%CV) determined at each concentration level should be within ±15% (±20% for LLOQ)
6.	Dilution integrity	Spiking of the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix.	To check whether dilution of samples were affecting the accuracy and precision.	Accuracy and precision should be within ±15%.

Sr. No.	Parameters	Experiment	Evaluation for	Acceptance criteria
7.	Recovery	Analyzed of 5 replicates of LQC, MQC and HQC extracted samples and 5 replicates of analyte at working standard concentration; Similarly determined recovery of IS	For %recovery by comparing response of extracted samples with that of unextracted samples	Recovery should be precise, consistent and reproducible
8.	Stability studies	Analyzed, Short-term stability QC samples kept at room temperature for 6 hours. Freeze-thaw stability QC samples subjected to three freeze and thaw cycles. Long-term stability QC samples stored in deep freezer at -20°C for 30 days.	Evaluation of %RE with respect to the fresh samples after back calculation from the CC	% RE should be within +15% and % CV should be less than 15%

Carry-over

Blank sample applied after the ULOQ sample (figure 4a and 4b) compared with the acceptance criteria for carry over. The calculated value of carryover for zolpidem tartrate and IS was 0%.

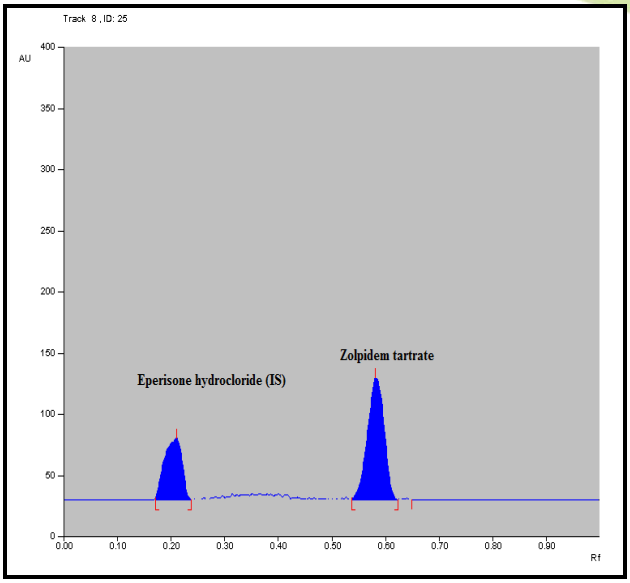

Figure 4a: Densitogram of ULOQ sample

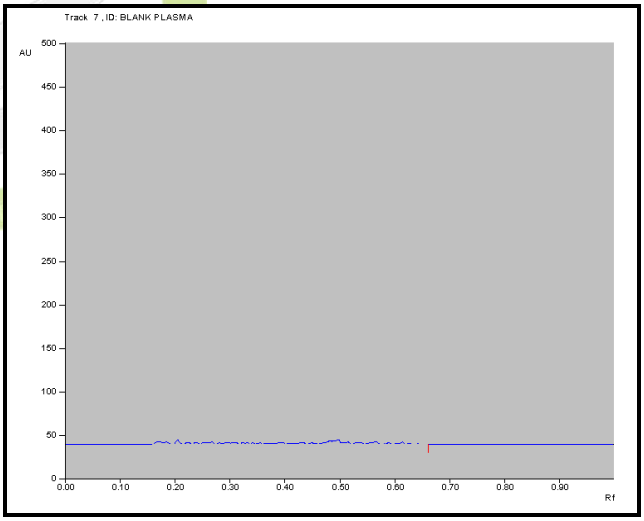

Figure 4b: Densitogram of blank sample applied after ULOQ

Lower Limit of Quantification (LLOQ)

LLOQ was found to be 100ng/mL with acceptable accuracy and precision (figure 3b).

Calibration Curve

Calibration curves were constructed over the linear range of concentration of 100ng/mL-

2500ng/mL on four different days. The ratio of area response for drug and IS was used for regression analysis. Each calibrationcurve was analyzed individually by using non-linear regression.

\Since, heteroscedasticity was observed by ordinary linear regression model, the best fit and least square residual model were used for calibration function by subjecting to weighted linear regression. The weighted $1/X^2$ linear regression model was selected. Table 4 gives the mean, standard deviation, % nominal and % coefficient of variation and it was found to be within acceptable limits of 15% accuracy and precision (20% for LLOQ).

Accuracy and Precision

Within-run and between run accuracy and precision results are shown in Table 5. The accuracy expressed as %RE and precision expressed as %CV were within acceptable limits at all concentration levels.

Dilution Integrity

Results obtained for dilution integrity are shown in table 6.

Recovery

Recovery of zolpidem tartrate and I.S. were found to be above 80% hence acceptable. Results obtained for recovery are shown in table 7.

Table 4: Results of linearity using calibration curve

Concentration (ng/mL)	Mean (Back calculated)	Standard deviation	%CV	% Nominal
100	90.56	11.28	12.46	90.56
500	536.14	19.12	3.56	107.22
900	864.78	6.14	0.71	96.08
1300	1297.17	6.14	0.47	99.78
1700	1826.75	38.10	2.08	107.45
2100	2159.15	40.62	1.88	102.81
2500	2481.21	45.27	1.82	99.24

Table 5: Results obtained for accuracy and precision

Parameters	Concentration (ng/mL)			
	LLOQ (100ng/mL)	LQC (500ng/mL)	MQC (1300ng/mL)	HQC (2100ng/mL)
Within run (n=5)				
Mean	111.20	512.02	1259.32	2128.23
% CV	6.50	7.29	7.20	4.01
% Nominal	111.20	102.40	96.87	101.34
Between run (n=20)				
Mean	115.4	501.81	1270.82	2097.77
% CV	0.626	2.17	2.37	1.18
% Nominal	115.4	100.36	97.57	99.89

Table 6: Results obtained for dilution integrity

Concentration level	Dilution factor (1:2) 6μg/mL	Dilution factor (1:4) 3μg/mL
Mean of back calculated concentration (μg/mL)	5.52	2.49
%RE	-8	-14
%CV	3.13	11.55
% Nominal	92.04	83.08

Table 7: Recovery results

QC samples	Mean peak area of standard solution	Mean peak area of extracted samples	Mean % recovery
LQC	727.06	621.46	85.47
MQC	1752.56	1477.567	84.30
HQC	2585.23	2422.73	93.71
IS	1684.61	1563.4	92.80

Table 8: Results of stability studies

Parameters	Short term stability		Freeze thaw stability		Long term stability	
	LQC (500 ng/mL)	HQC (2100 ng/mL)	LQC (500 ng/mL)	HQC (2100 ng/mL)	LQC (500 ng/mL)	HQC (2100 ng/mL)
Mean	532.84	2161.96	504.64	2241.38	501.96	2137.96
Standard deviation	16.24	24.28	40.62	84.27	51.67	82.13
% CV	3.04	1.12	8.04	3.75	12.64	5.76
% Nominal	106.56	102.95	100.92	106.73	102.34	105.91
% Deviation	-3.19	-0.43	-1.45	8.38	-5.41	-7.26

Stability Studies

The results of stability of zolpidem tartrate are summarized in table 8. From the results, it was concluded that all the samples were stable under specified conditions and within the acceptable limits of accuracy and precision. There was no significant decrease in the concentration of drug in stability samples when compared with freshly prepared samples. Less than 10 % deviation was seen for the stability samples on comparison with fresh samples.

CONCLUSION

The proposed work was carried out with an objective of developing simple, selective, accurate and precise bioanalytical method for estimation of zolpidem tartrate from human plasma. HPTLC method for estimation of zolpidem tartrate from human plasma was developed and validated as per recommended regulatory guidelines. The developed bioanalytical HPTLC method was found to be specific, linear, accurate, precise and robust; hence this bioanalytical method can be effectively applied for analysis of real samples, for performing BA-BE studies of the new generic formulation of zolpidem tartrate.

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