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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND RP HPLC METHOD FOR THE ESTIMATION OF ESZOPICLONE BULK AND TABLETS

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Abstract

Objective: Objective of the present analytical research work was to develop and validate Spectrophotometric method and Reverse Phase High Performance Liquid Chromatographic method (RP-HPLC Method) for the Eszopiclone bulk and tablets dosage form.

Methods: A spectrophotometric method and a RP-HPLC method have been developed and validated for estimation of ESZ in pharmaceutical oral dosage form.

Method A (RP-HPLC Method): The RP-HPLC Method for Eszopiclone was developed using Shimadzu HPLC, LC-10, temperature maintained 25^{0}C, phenorex Gemini C18 (250 mm × 4.60 mm × 5µm), as stationary particle, isocratic mode. MeOH: Water (80:20v/v) as mobile phase. Mobile phase was maintained at a flow rate of 1.0 ml/min and detection was carried out at 305 nm.

Method B (UV SPECTROMETRY Method): The stock and working standard solutions of the drugs were prepared in methanol. Standard solutions were scanned over the range of 400-200 nm in spectrum mode of spectrophotometer at medium scanning speed using UV spectrophotometer 2450, SHIMADZU. The maximum absorbance for Eszopiclone was found at 305 nm. Both the methods were validated in accordance with ICH guidelines

Results: Eszopiclone was found to be linear in the concentration range of 4 - 24 µg/ml for spectrophotometric method and 5-30 µg/ml for RP-HPLC method. Retention time was found to be 5.38 min for Eszopiclone. The amount of Eszopiclone in marketed formulation by spectrophotometric method was found to be 100.02 %, the amount of Eszopiclone in marketed formulation by RP-HPLC method was found to be 100.03 %.

Interpretation and Conclusion: Results of assay and validation study were found to be satisfactory. So, the methods can be successfully applied for the routine analysis of Eszopiclone.

***Keywords*:** Eszopiclone, RP-HPLC, UV Spectrometry, ICH Guidelines

1. Introduction

Insomnia is a sleep disorder in which there is an inability to fall asleep or to stay asleep as long as desired. Insomnia is very common and occurs in 30% to 50% of the general population. 10% of the population may suffer from chronic (long-standing) insomnia. Insomnia affects people of all ages including children, although it is more common in adults and its frequency increases with age.[1] In general, women are affected more frequently than men. There are various symptoms of insomnia like difficulty falling asleep despite being tired,walking up frequently during the night,trouble getting back to sleep when awakened,difficulty with memory,relying on sleeping pills or alcohol to fall a sleep,walking up too early in the morning,daytime drowsiness, fatigue or irritability,difficulty concentrating during the day. Eszopiclone is one of the drug is available in the market for the treatment of insomnia.[2]

Eszopiclone is Calcium chnnel blocker acts on benzodiazepine binding site situated on GABAA neurons as an agonist. Cytochrome P450 (CYP) isozymes CYP3A4 and CYP2E1 are involved in the biotransformation of Eszopiclone. γ- Amino butyric acid (GABA) is the most important inhibitory neurotransmitter in the mammalian brain and localizes to approximately 30% of CNS synapses. This inhibitory neurotransmitter is of particular interest because most therapeutically useful hypnotic drugs work by selectively affecting GABA receptors. A number of classes of GABA receptors including the GABAA, GABAB, and GABAC receptors have been characterized in the CNS of several species, including man. The distribution of GABA receptor types varies throughout the CNS. The GABAA receptor is the site of action of Eszopiclone. It modulates GABAnergic function through different GABAA receptor sub-types, defined by the subunits that participate in the receptor assembly. Most GABAA receptors consist of α, β, and γ subunits which contain multiple isoforms or variants: α1–α6, β1–β3, and γ1–γ3.[3]

Literature survey reveals that only a few analytical methods are reported for the estimation of Eszopiclone. In this study efforts were made to develop a simple, easy and economic UV-Visible Spectrophotometry and HPLC methods for the estimation of Eszopiclone. The developed method was optimized and validated as per the guidelines of International conference on Hormonization (ICH) and demonstrated excellent specificity, linearity, precision and accuracy for Eszopiclone. Validation is a process of establishing documented evidence, which provides

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a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products.[4, 5, 6]

2. Materials and methods

2.1 Preliminary analysis of drug

Eszopiclone (ESZ) is official in United States Pharmacopoeia (USP). It is in Authorized USP Pending Monograph Version 1. Hence, Preliminary analysis of ECZ was performed according to USP.

2.1.1 Description

The sample of ECZ was observed for its color and texture.

2.1.2 Solubility

The sample of ECZ was taken in test tubes and observed for solubility in various solvents like methyl chloride, diluted mineral acids water, alcohol and water.

2.1.3 Water Determination

Water content can be determined using Karl Fischer Titrimeter. Karl Fischer reagent is standardize with sodium tartrate, then it is allowed to titrate with the known amount of sample i.e. ESZ and then once the color change is observed, it will indicate the end point of the titration and this in turn will give the amount of moisture present in the sample. Moisture/water content can be determine using following formula,

$$\text{Water content} = \frac{\text{Burette reading} \times \text{Karl Fisher factor} \times 100}{\text{Weight of Sample (mg)}}$$

2.2 Chemicals and Reagents (HPLC)

Analytically pure samples of Eszopiclone were kindly supplied by Sun Pharmaceuticals Ltd, Vapi, Gujarat, India. Zopipure (Marketed Formulation), Water (HPLC Grade), MeOH (HPLC Grade) Themis laboratories Pvt.Ltd (Mumbai,India), were used for the method development.

2.2 Instrument Used

Electronic Weighing Balance (Tapson's Analytical Balance), Ultrasonicator (Tapson's TP-101), Cellulose Acetate Filter, 0.45 µm (Nylon 66), HPLC System (Shimadzu)

2.3 Selection of Mobile Phase

The selection was made on the basis of literature survey. After assessing the solubility of drug in different solvents as well on the basis of literature survey, Methanol and Double RO water were selected as a first choice.

2.4 Selection of column (stationary phase)

To get well resolved, symmetric peak with highest no. of theoretical plates, the solution of ESZ was analyzed using different column as a stationary phase like Phenyl, C18, Amino column.

2.5 Selection of Analytical Wavelength

To investigate the appropriate wavelength for determination of ESZ, the solution of the same in the MeOH were scanned separately by UV–Visible spectrophotometer in the range of 190-400 nm and the spectrum were recorded.

2.6 Preparation of Mobile Phase

***Mobile Phase A*:** HPLC grade MeOH was degassed in sonicator for 15 min.

***Mobile Phase B*:** Double RO water

2.7 Preparation of Standard Stock Solution

Standard stock solution was prepared by dissolving 10 mg of ESZ in 100 ml methanol that gives concentration of 100 □g/ml of ESZ and labeled as Standard stock ESZ.

2.8 Preparation of Calibration Curve for ESZ

2.9 Analysis of Zopipure I (Sun Pharmaceutical Ltd)

To determine the content of ESZ in conventional tablets (Label claim 1 mg ESZ per tablet); the twenty tablets were weighed, their mean weight determined and they were finely powered and powder equivalent 1.0 mg ESZ was transferred into a 100 mL volumetric flask containing 40 mL methanol, sonicated for 30 min and diluted to 100 mL with methanol (10 µg/mL). The resulting solution was filtered, using 0.22 µm filter (Millifilter, Milford, MA) and injected into system. The amount of ESZ was determined. The assay procedure was repeated for six times and Calculated using following equation.

$$Ct = \frac{Rt \times Cs}{Rs}$$

Where, Ct and Cs = Concentration of Sample and Standard Solution, respectively.

Rt and Rs = Peak Area for Sample and Standard Solution, respectively.

2.10 Validation of RP-HPLC Method

2.10.1 Accuracy

Accuracy of the method was assessed by standard addtition method at three different concentration levels i.e. 80%, 100, 120%. From sample stock solution ESZ (100 µg/ml), pipette out 10 ml to each of four different 100 ml volumetric flask and add to it 0.0 ml, 8.0 ml, 10.0 ml and 12.0 ml of standard stock solution ESZ (100 µg/ml) and make up the volume with MeOH. The % Recoveries was calculated by applying regression equation.

2.10.2 Precision

The precision of an analytical method was studied by performing repeatability and intermediate precision.

2.10.3 Repeatability

Suitable aliquot from standard stock solution ESZ (100 µg/ml) 1 ml was pipetted in 10 ml volumetric flask and make up the volume to get final concentration of 10 µg/ml and analyzed six times on the same at optimized chromatographic conditions.

2.10.4 Intermediate Precision

2.10.4.1 Intra-day Precision

Intra-day precision was determined by analyzing the standard solutions of ESZ (10, 15, 20 µg/ml) and at three different time intervals on same day.

2.10.4.2 Inter-day Precision

Inter-day precision was determined by analyzing the combined standard solutions of ESZ (10, 15, 20

µg/ml) on three consecutive days. The results were reported in terms of % RSD.

2.10.5 Linearity and Range

The linearity of analytical method for ESZ was determined by studying standard calibration curves. The range of analytical method was decided from the interval between upper and lower level of calibration curves by plotting the log curve.

2.10.6 Limit of Detection and Limit of Quantitation

Detection limit and quantitation limit were determined based on the standard deviation of y-intercepts of six calibration curves and average slope of six calibration curves.

$$LOD = 3.3 \times \frac{\text{Standard deviation of intercept}}{\text{Slope}}$$

$$LOD = 10 \times \frac{\text{Standard deviation of intercept}}{\text{Slope}}$$

2.10.7 Robustness

Standard stock solution of ESZ (100 µg/ml)) were used and analyzed at different flow rate (0.9, 1.00, 1.1 ml/min) and at different mobile phase ratio (79:21, 80:20, 81:21 v/v) separately.

2.10.8 System Suitability

Standard solution of ESZ (100 µg/ml) was prepared and analyzed. Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to see that whether they complies with the recommended limit or not.

2.10.9 Specificity and Selectivity

The analytes should have no interference from other extraneous components and be well resolved from them. Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix. The method is quite selective. There was no other interfering peak around the retention time of ESZ; also the base line did not show any significant noise.

2.10.10 Ruggedness

Ruggedness of the method was checked by two different analysts keeping same experimental and environmental conditions. An appropriate concentration 10 µg/ml of ESZ was subjected to analysis and concentration was determined. This procedure was repeated six times.

2.11 Chemicals and Reagents (UV Visible Spectrophotometric)

Eszopiclone (Standard)*,Zopipure (Marketed Formulation) [Label claim: 1mg of ESZ mg per tablet],MeOH (AR Grade),Chloroform (AR Grade),0.1M NaOH,0.1M HCl, *Eszopiclone was provided by Sun pharma, Vapi, Gujarat.

2.12 Instruments Used

Electronic Weighing Balance (Tapson's Analytical Balance), Ultrasonicator (Tapson's TP-101)

UV–Visible Spectrophotometer (UV spectrophotometer 119, Systronics, Software Version 1)

2.13 Selection of Solvent

Solutions of ESZ (100 µg/ml) was prepared in different solvents like 0.1M HCl, 0.1M NaOH, methanol and Chloroform. These solutions were scanned in UVVisible Region (200 nm to 800 nm) and intensity of absorption and wavelength of absorption were studied.

2.14 Preparation of Standard Stock Solution

Standard stock solution was prepared.

2.15 Selection of Wavelength Range

From the stock solutions, 1.0 ml of ESZ was transferred to 10 ml volumetric flask and the volume was adjusted to the mark with MeOH to obtain Strength 10µg/ml. The solution was scanned in the UV range 200-400 nm.

2.16 Preparation for Calibration Curve

Calibration curve were prepared and graph was plotted.

2.17 Determination of E (1%, 1 cm) and Molar Absorptivity

Aliquot portions of ESZ stock standard solution were transferred in to five different 10 ml volumetric flasks; diluted with same solvent to obtain concentration of 16 µg/ml. The absorbance of each solution was measured at 305.0 nm. A (1%, 1cm) values of drugs were calculated using following formula, A (1%, 1cm) = Absorbance/Concentration (g/100ml)

Molar absorptivity was determined from following equation.

$$\text{Molar absorptivity} = \frac{\text{Absorptivity} \times \text{Molecular weight}}{10}$$

2.18 Analysis of zopipure

For analysis of commercial formulation, twenty tablets were weighed, average weight determined and crushed into fine powder. An accurately weighed quantity of powder equivalent to 1 mg of ESZ was transferred into 100 ml volumetric flask containing 30 ml methanol (50%,*v/v*), shaken manually for 10 min, volume was adjusted to mark with same solvent and filtered through Whitman filter paper no. 41. The absorbance of sample solution was recorded at recorded at 305 nm.

2.19 Validation of Spectrophotometric Method

2.19.1 Accuracy

Accuracy of the method was assessed by standard addtition method at three different concentration levels i.e. 80%, 100, 120%. From sample stock solution ESZ (100 µg/ml), pipette out 10 ml to each of four different 100 ml volumetric flask and add to it 0.0 ml, 8.0 ml, 10.0 ml and 12.0 ml of standard stock solution ESZ (100 µg/ml) and make up the volume with MeOH. The % recovery was then calculated by using formula

% Recovery = A – B/ C, Where,

A = Total amount of drug estimated

B = Amount of drug found on pre analysed basis

C = Amount of Pure drug added

2.19.2 Precision

The precision of an analytical method was studied by performing Repeatability and Intermediate precision.

2.19.3 Repeatability

Suitable aliquot from standard stock solution ESZ (100 µg/ml) 1 ml was pipetted in 10 ml volumetric flask and make up the volume to get final concentration of 10 µg/ml and analyzed six times on the same at optimized chromatographic conditions The standard deviation and % Relative standard deviation were also calculated.

2.19.4 Intermediate precision

2.19.4.1 Intra-day Precision

Intra-day precision was determined by analyzing the 12, 16, 20 µg/ml of ESZ for three times in the same day.

2.19.4.2 Inter-day Precision

Inter-day precision was determined by measuring the the 12, 16, 20 µg/ml of ESZ for three consecutive days.

2.19.5 Linearity and Range

The linearity of analytical method for ESZ was determined by studying standard calibration curves. The range of analytical method was decided from the interval between upper and lower level of calibration curves by plotting the log curve.

2.19.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Detection limit and quantitation limit were determined based on the standard deviation of y-intercepts of six calibration curves and average slope of six calibration curves.

$$LOD = 3.3 \times \frac{\text{Standard deviation of intercept}}{\text{Slope}}$$

$$LOD = 10 \times \frac{\text{Standard deviation of intercept}}{\text{Slope}}$$

2.19.7 Ruggedness

Ruggedness of the method was checked by two different analysts keeping same experimental and environmental conditions. An appropriate concentration 10 µg/ml of ESZ was subjected to analysis and concentration was determined. This procedure was repeated six times.[5-13]

3. Results and discussion

3.1 Preliminary analysis of drug

Preliminary analysis of Eszopiclone such as description, solubility, identification test and assay were performed according to available literature survey and complied with USP. A white to slightly fine powder was found which was complied with USP. Drug was soluble in methanol, methylene chloride and insoluble in water and alcohol. Water content was found 0.2%.

3.2 High Performance liquid Chromatographic Method

3.2.1. Method A: Reverse Phase HPLC method

By the literature survey RP-HPLC method for estimation of Eszopiclone was reported using RP8 column (150mm×4.6mm i.d., 3.5m particle size) column. Initially, RP8 column was tried to achieve good peak, but it was not obtained thus this prompted to use Phenomenex Gemini C18 column (250 mm x 4.6.0 mm, 5 µ), thus Phenomenex Gemini C18 column was selected. The observed for ESZ were min 5.38 min. Thus the mobile phase containing mixture MeOH: Water (80:20 v/v), at a flow rate of 1 ml/min with UV detection at 305 nm, using Phenomenex Gemini C18 column (250 mm x 4.6.0 mm, 5 µ) as a stationary phase was finalized. This also improved the column efficiency and ease of separation. **Table 1, Figure1, 2, 3**

3.2.2 Selection of Analytical Wavelength

The standard solutions of ESZ (100 µg/ml) in mobile phase were scanned in the UV region of 190 - 400 nm and the overlain spectra were recorded. It was observed that ESZ drugs showed the absorbance at 305 nm. So, the wavelength of detection used was 305 nm.

3.2.3 Linearity Study

ESZ was found to be linear in the concentration range of 5-30 µg/ml. **Table 2 and Table 3**

3.2.4 Assay of Marketed Formulation

Amount of drugs present in the marketed formulation (Zopipure I, Sun Pharmaceutical Ltd.) using equations mentioned in the section no. 4.2.8.Amount of ESZ found in the range from 100.03% and SD ± 0.70. **Table 4**

3.2.5 Validation Parameters

This method was validated in accordance to ICH guidelines. Percentage of recoveries of ESZ was found in the range from 99.90 – 100.09%. Precision of the method was determined by % RSD found among intra-day precision, inter-day precision, repeatability. LOD and LOQ of ESZ were found to be 0.310 and 0.572µg/ml, respectively. For robustness study, the effect of change in of mobile phase, mobile phase ratio and flow rate (1.0 ± 0.2 ml/min) on the Mean peak area, % RSD and % Assay were studied. Standard solutions of ESZ (100 µg/ml), was prepared and analyzed at different mobile phase ratio (90:10, 80:10, 50:50v/v) and at different flow rate (0.99, 1.00, 1.1 ml/min). Percentage RSD of each peak in all variables was found to be less than 3 %. **Table 5, 6, 7, 8, 9, 10**

3.3 UV-Visible Spectrophotometric Methods

3.3.1 Selection of solvent

The spectrum of the drug in different solvents like 0.1M HCl, 0.1M NaOH, methanol and chloroform were studied carefully during the development of UV-Visible spectrophotometric method. But among all these spectra's of ESZ obtained from various solvents, the spectrum obtained with 50%v/v methanol was found to be better as ESZ was stable in solvent, shows very distinct and clear absorbance at 305 nm. **Figure 4, 5**

3.3.2 Single Point Method

As traditional UV spectrophotometric method development was found suitable for estimation of this drug from marketed formulation. So, estimation was done with the help of spectrum mode of analysis. In this method methanol was used as a solvent while ESZ absorbed at 305nm and wavelength range of 400 nm to 200 nm was selected for the absorbance mode of analysis at medium scanning speed.

3.3.3 Linearity Study

Standard solution having concentration range of 4, 8, 12, 16, 20, 24 and µg/ml of ESZ was prepared. Absorbances of these solutions were recorded at 305.0 nm. Calibration curve was plotted, absorbance *vs* concentration. **Table 11**

3.3.4 Assay of Marketed Formulation

Using this method, the marketed formulation (Zopipure I, Sun Pharmaceutical Ltd.) was analyzed. Sample solution containing 10 µg/ml. The amount of drug present in the marketed formulation was calculated .The mean % assay of ESZ was found to be 100.02%. **Table 12, 13, 14**

3.3.5 Validation Parameters

Validation of the method was performed in accordance to ICH guidelines. Accuracy of the method was determined at 80%, 100% and 120% level by standard addition method and percentage recovery ESZ were found to be in the range of 99.54 – 100.10 %. Precision of the method was determined by % RSD of intra-day precision, inter-day precision, and repeatability. It was found to be less LOD and LOQ of ESZ was found to be 0.429 and 1.310 µg/ml, respectively. **Table 15,16,17,18**

4. Conclusion

In the present investigation, the developed and validated, UV Spectrophotometric method were found to be simple, economical and rapid method. RP-HPLC was found to more precise, accurate, rugged and robust for determination of Esczopiclone. The excipients usually present in the pharmaceutical formulation did not interfere with determination of Eszopiclone. Developed method can be successfully used in laboratory to measure the concentration of API in specific dosage form. This method is also beneficial for the formulation and development department. These methods are always useful for analysis, purity testing and assay. The consumption of time and chemicals is less as compare to other tedious method. This is new concept for the validation of method development and method transfer in pharmaceutical companies. The results and the statistical parameters demonstrate that the proposed UV spectrophotometric and RP-HPLC method is simple, rapid, specific, accurate and precise.

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References

1. Tripathi K, Essential of medical pharmacology, 4th ed.,Jaypee brother, Delhi, 2001, pp.360-368.
2. R S Satoskar, S D Bhandarkar, Nirmala N.Rege. *Phrmacology and pharmaco therapeutics* , Popular R Prakashan,3rd Mumbai, 2001,p.110-115.
3. Hotha K, Vijaya Bharathi D, Jagadeesh B, Ravindranath L, Jaya Veera K and Venkateswarulu V. A rapid LC-MS/MS method for quantitation of eszopiclone in human plasma: application to a human pharmacokinetic study. *Biomed chromatogr*. 25(5) (2011) 220-227.
4. Beckett AH, Stenlake JB. Practical Pharmaceutical Chemistry, 4th ed. Part II CBS Publishers and Distributors, Delhi, 2007, pp. 274-325.
5. Validation of analytical procedures: text and methodology, in: International Conference on Harmonization (ICH), Q2(R1), IFPMA, Geneva, Switzerland, 2005.
6. Mistri H, Jangid A, Pudage A and Shrivastav P. HPLC-ESI-MS/MS validated method for simultaneous quantification of zopiclone and its metabolites, Ndesmethyl zopiclone and zopiclone-N-oxide in human plasma. *J Chromatogr B Anlayet Technol Biomed Life Sci* 864 (1-2) (2008) 137-48.
7. Dhaneshwar S, Bhusari V. Development of a Validated Stability Indicating HPLC assay method for Eszopiclone. *Int J of Chem Tech Res.* 3(2) (2011) 680-89.
8. Bouklouze A, Vire J, Quarin G, Kauffmann J. Quantitative analysis of zopiclone in tablets using ion-selective electrode and polarographic methods. *J Chromatogr* 6 (11) (2005) 1045-50.
9. Paw B, Misztal G. Determination of zopiclone in tablets by HPLC and UVspectrophotometry . *J Chromatogr* B 23(5) (2000) 819–823.
10. Stanke F, Jourdil N, Lauby V, Bessard V. Zopiclone and Zolpidem in Human Plasma by High Performance Liquid Chromatography with Photo-Diode- Array. *J Liq Chromatogr* 23(1996) 19(16).
11. Kataev S, Zelenina B, Khomov Y, Koksharova N, Daiekh K. Determination of zopiclone in urine by gas chromatography-mass spectrometry. *J Anal Chem* 62(5) (2007) 458-462.
12. Mannaert E , Tytgat J, Daenens P. Detection of 2-Amino-5-Chloropyridine in Urine as a Parameter of Zopiclone Intake using HPLC with Diode Array Detection. *J Anal Toxicol* 21 (3) (1996) 208-212.
13. A.A. Shirkhedkar, S.J. Surana, Quantitative determination of levofloxacin hemihydrate in bulk and tablets by UV-Spectro- photometry and first order derivative methods, *Pak. J. Pharm. Sci.* 22 (3) (2009) 301–302.

Table 1 Observation and remarks of mobile phase Optimization of Chromatographic Conditions

Mobile Phase Composition	Inference	Conclusion
MeOH : Water (10 : 90 v/v)	Rt of Esz greater than 10	Mobile phase was not satisfactory
MeOH : Water (20 : 80v/v)	Rt of Esz was around 9.10	Mobile phase was not satisfactory
MeOH : Water (50 : 50v/v)	Rt of Esz was around 7	Mobile phase was not satisfactory
MeOH : Water (90 : 10 v/v)	Asymmetry was less.	Mobile phase was not satisfactory
MeOH : Water (80 : 20 v/v	Rt of Esz was around 5.38 , Assymmetry was good	Mobile phase was suitable (optimized)

Table 2 Results of Calibration Curve of ESZ

S.No	Concentration of ESZ [µg/mL]	Area Mean	% RSD
1	5	142161.6	0.80
2	10	295647.2	0.60
3	15	423567.2	0.30
4	20	571901.3	0.85
5	25	702345.7	0.79
6	30	842356.1	0.32

Table 3 Linear Regression Analysis of Calibration Curves for ESZ

Linearity Range (µg/ml)	5-30
Slope	27825
Intercept	9389.6
Correlation Coefficient (r2)	0.9995
LOD (µg/ml)	0.310
LOQ (µg/ml)	0.572

Table 4 Assay Results of Zopipure RP-HPLC Method

Label Claim [mg]	Amount found [mg]	[%] of Assay
10	10.02	100.02
10	10.08	100.08
10	10.10	101.10
10	9.97	99.70
10	9.93	99.31
10	10.01	100.01

Table 5 Results of Accuracy for RP-HPLC Method

Initial Amount [µg/mL]	Amount added [µg/mL]	Amount recovered ± S.D [µg/mL, n=3]	% Recovery	% RSD
10	0	9.99 ± 0.06	99.90	0.52
10	8	17.97 ± 0.06	99.83	0.82
10	10	19.96 ± 0.05	99.80	0.65
10	12	22.02 ± 0.05	100.09	0.40

Table 6 Results of intra-day precision and inter-day precision

Concentration [µg/ml]	Intra-day Precision		Inter-day Precision	
	Mean	% RSD	Mean [n= 3]	% RSD [n= 3]
10	10.13	1.85	10.12	0.43
15	14.97	1.37	14.90	1.51
20	19.73	1.48	19.73	1.10

Table 7 Results of Repeatability Study for ESZ

Mean	279540.2
S.D	376.62
% RSD	0.65

Table 8 Result of Robustness Study: Variation in Mobile phase Ratio

Percentage Methanol in Mobile phase (v/v)	RT	K'	T
81	6.05	1.20	1.47
80	5.38	0.90	1.40
79	4.01	0.73	1.45
Mean ± SD	5.14 ± 0.60	0.94 ± 0.20	1.44 ± 0.04

Table 9 Results of Ruggedness

Analysts [n=3]	Amount found of ESZ [%]	%RSD
I	100.04	0.34
II	100.05	0.40

Table 10 Results of System Suitability Parameters

Analyte	Retention time (min)	Tailing Factor	Theoaretical plates (N)	Capacity Factor
ESZ	5.3	1.40	4122	0.92

Table 11 Results of Calibration Curve of ESZ

S. No	Concentration of ESZ [µg/mL]	Absorbance Mean	% RSD
1	4	0.143±0.0011	0.68
2	8	0.285±0.0011	0.34
3	12	0.416±0.0009	0.20
4	16	0.554±0.0011	0.17
5	20	0.680±0.0012	0.15
6	24	0.805±0.0011	0.12

Table 12 Linear Regression Analysis of Calibration Curves for ESZ

Parameters	ESZ
Linearity Range (µg/ml)	4-24
Slope	0.0331
Intercept	0.172
Correlation Coefficient (r2)	0.9995
LOD (µg/ml)	0.429
LOQ (µg/ml)	1.310

Table 13 Analysis of ESZ in bulk sample

Amount Taken (µg/ml)	Amount Found (µg/ml)	Amount Found ± SD (%, n = 5)	% R.S.D.
10	9.98	99.8±0.26	0.26

Table 14 Analysis of Zopipure –I (ESZ tablets)

Sample	Label claimed	%Label claim ± SD	%RSD
1	10	100.02±0.47	0.47

Table 15 Accuracy of ESZ

Initial Amount [µg/ml]	Amount added [µg/ml]	Amount Recovered	% Recovered [µg/ml]	% R.S.D
10	8	17.94	99.54	0.18
10	10	20.00	100.02	0.23
10	12	12.05	100.10	0.13

Table 16 Results of intra-day precision and inter-day precision

Concentration [µg/ml]	Intra-day Precision		Inter-day Precision	
	Average potency	% RSD [n= 3]	Average potency	RSD % [n= 3]
10	99.70%	0.21	99.33%	0.39
15	99.85%	0.40	99.69%	0.47
20	99.60%	0.46	99.90%	0.55

Table 17 Results of Repeatability Study for ESZ

Mean	279540.2
S.D	376.62
% RSD	0.44

Table 18 Results of Ruggedness

Analysts [n=3]	Amount found of ESZ [%]	%RSD
I	100.04 ± 0.42	0.42
II	100.05 ± 0.23	0.23

Figure 1 HPLC chromatogram of Blank (diluents) MeOH: Water (80:20, v/v)

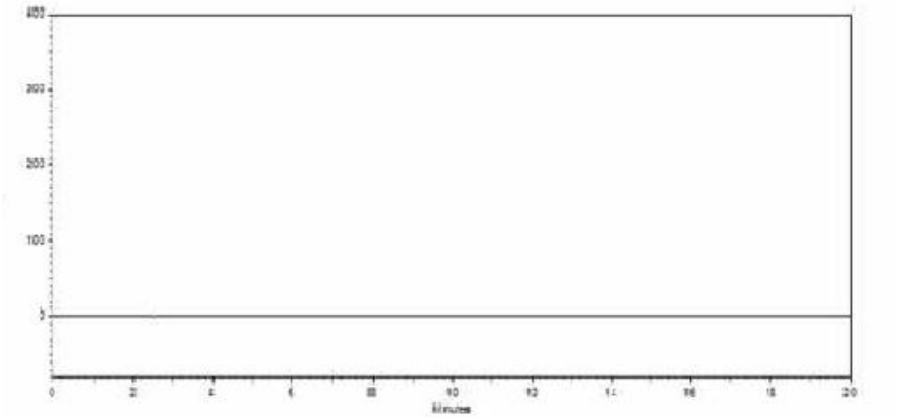

Figure 2 HPLC chromatogram of ESZ standard (100 µg/ml) mobile phase at flow rate of 1 ml/min, at 305nm,

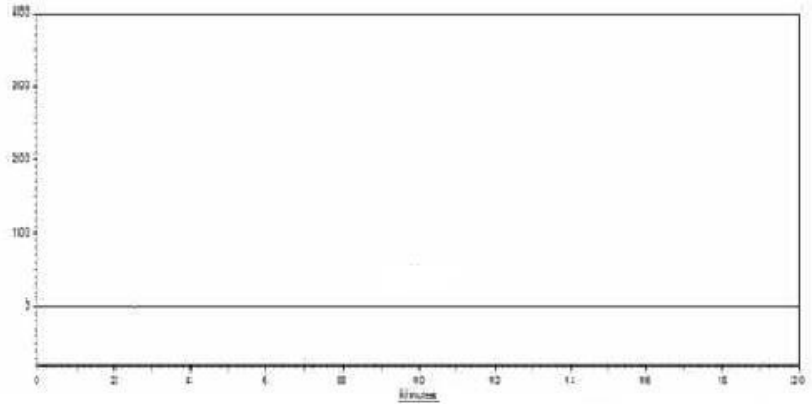

Figure 3 HPLC chromatogram of sample ESZ (10 µg/ml) in the optimized mobile phase at flow rate of 1 ml/min, at 305nm, C18 colum

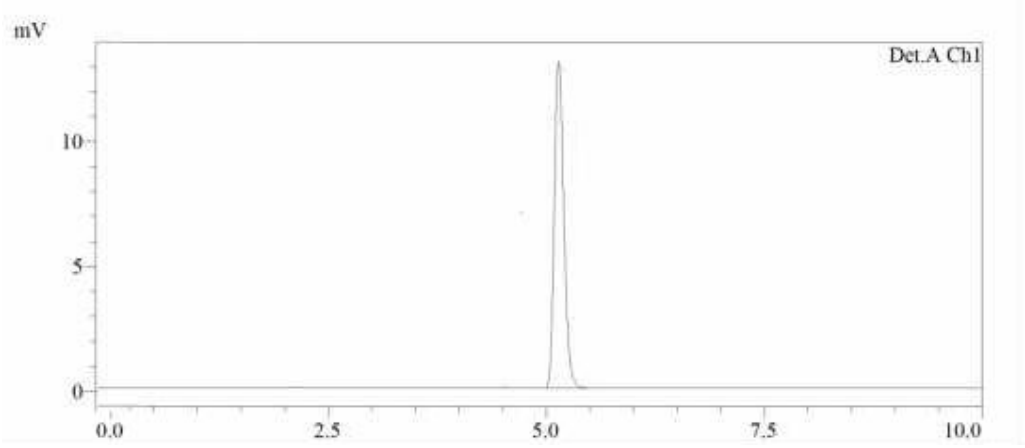

Figure 4 Spectrum of eszopiclone in 50% v/v methanol

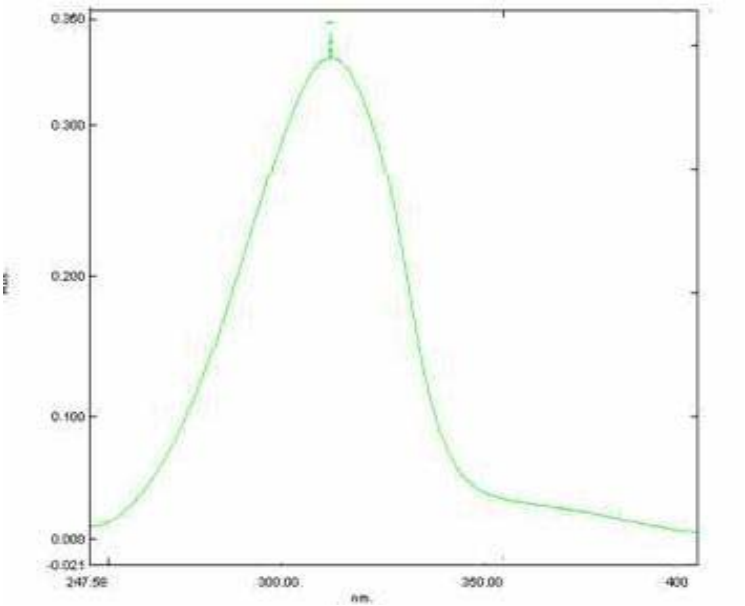

Figure 5 Overlain spectra of ESZ (4 - 24 µg/ml) in methanol