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METHOD DEVELOPMENT AND VALIDATION OF ZOPICLONE IN BULK AND TABLET DOSAGE FORM USING RP-HPLC

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Gradient elution, RP-HPLC method

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ABSTRACT: Zopiclone belongs to a class of medicines commonly called Z-drugs. It is a novel hypnotic agent used in the treatment of insomnia. Its mechanism of action is based on modulating benzodiazepine receptors. A Reverse Phase High Performance Liquid Chromatography method was developed and validated for the quantification of zopiclone in bulk and tablet dosage form. Chromatography was achieved using a Waters X Bridge C18 column (50 x 2.1 mm, 5µ) eluted with a mixture of mobile phase composed of ammonium bicarbonate (5mM, neutral) and acetonitrile in a gradient mode at a flow rate of 1 ml/min. The detection of eluent from the column was detected using photo diode array detector (PDA) at 214nm. The stability indicating assay method was developed and validated as per the ICH guidelines using the required parameters. Linearity was observed within the concentration range of 50-300µg/ml with coefficient of correlation 0.999. The LOD and LOQ were 9.416µg/ml and 28.534µg/ml, respectively. The mean recovery was found to be 100.1%.

INTRODUCTION: Zopiclone **(Fig. 1)** is 6-(5-chloropyridin-2-yl)-7-oxo- 6, 7 - dihydro - 5H-pyrrolo[3,4-b]pyrazin-5-yl-4-methylpiperazine-1-carboxylate[1]. Zopiclone is a nonbenzodiazepine hypnotic from pyrazolopyrimidine class used in the treatment of insomnia. While Zopiclone is a hypnotic agent with a chemical structure unrelated to benzodiazepines, barbiturates, or other drugs with known hypnotic properties, it interacts with the gamma-aminobutyric acid-benzodiazepine ($GABA_BZ$) receptor complex. Zopiclone exerts its action by binding on the benzodiazepine receptor complex and modulation of the $GABA_BZ$ receptor chloride channel macromolecular complex.

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Both zopiclone and benzodiazepines act indiscriminately at the benzodiazepine binding site on α1, α2, α3 and α5 $GABA_A$ containing receptors as full agonists causing an enhancement of the inhibitory actions of GABA to produce the therapeutic (hypnotic and anxiolytic) and adverse effects of zopiclone [2-4].

Its molecular formula is $C_{17}H_{17}ClN_6O_3$ and its molecular weight is 388.808 g/mol. It has following structural formula:

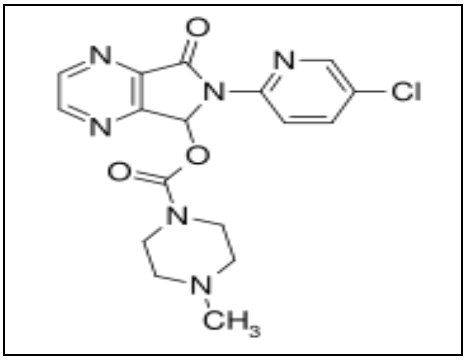

FIG. 1: MOLECULAR STRUCTURE OF ZOPICLONE

A simple and sensitive method for routine usage is generally desirable. Therefore, a need of simple, reliable, inexpensive, and accurate stability indicating method for analysis of Zopiclone as bulk or as tablet dosage form is always welcomed. The present study was aimed to develop and validate stability indicating method for the quantification of zopiclone in its tablet dosage forms.

MATERIALS AND METHODS: [5-14]

Chemical and Reagents:

The separation of the analyte was done by using Waters e2695 HPLC instrument with a Waters X Bridge C_{18} column (50 x 2.1 mm, 5µ) column. The instrument was equipped with a pump (2695), injector, PDA Detector (2996) and column oven. Data acquisition was done by using Empower software. Degassing of the mobile phase was done by using an ultrasonic bath sonicator. A Mettler Toledo (XS 205 dual range) electronic balance was used for weighing the materials. Class 'A' Borosil glassware were employed for volumetric and general purpose in the study. The reference sample of Zopiclone was gifted by Jubilant Life Sciences, Noida. The tablets of Zopiclone (The branded formulation of Intas Pharmaceuticals) were procured from the local market. Ammonium bicarbonate (AR grade, Merck), Acetonitrile (HPLC grade, Sigma Aldrich), Methanol (HPLC grade, Sigma Aldrich), water (Milli-Q / HPLC grade) were used.

Chromatographic Conditions Table 1:

Preparation of 5mM Ammonium bicarbonate solution:

385 mg Ammonium bicarbonate was weighed and dissolved in 1000 ml Milli-Q water, mixed well, filtered and degassed.

Mobile Phase:

Buffer and Acetonitrile were taken in separate bottles, filtered through 0.2µ Nylon membrane filter paper, sonicated, degassed and mixed automatically as per the method.

Diluent:

Milli-Q water: Acetonitrile (ACN) in a ratio (30:70) was mixed well, sonicated and degassed. It was also used as blank solution.

Preparation of Standard Solution:

About 5 mg of Zopiclone standard was weighed accurately and transferred to a 10ml volumetric flask. A little quantity of diluent was added to dissolve; sonicated and degassed. The volume was made up to 10 ml and sonicated (500ppm). Further 2 ml of the above solution was diluted to 5 ml with diluents (200ppm).

Preparation of Sample (Tablet Formulation):

10 tablets of Zolpidem, each containing 7.5 mg of Zopiclone, were weighed and powdered. To prepare 200µg/ml concentration of sample solution, a quantity of powder equivalent to 10 mg (110.005 mg) was weighed approximately and transferred to a 10 ml dried volumetric flask. The sample was initially dissolved in diluent and sonicated for 15 min. The volume was made up to 10ml and filtered through 0.2µm Nylon filters. Then 2 ml of this solution was further diluted to 5 ml with same diluent to get the final concentration of 200µg/ml and sonicated for 15 min again.

TABLE: 1 OPTIMIZED CHROMATOGRAPHIC CONDITIONS

S. No.	Parameter Optimized	Optimized Condition
1	Instrument (HPLC)	Waters e2695 PDA Detector
2	Column	Waters X-Bridge C18 (2.1 x 50 mm, 5µm)
3	Mode	Gradient
4	Mobile phase	Ammonium bicarbonate: Acetonitrile
5	Column Oven	25 ºC
6	Auto sampler Temperature	15º C
7	Flow rate	1 ml/min
8	Detector	Photodiode array
9	Temperature	Ambient room temperature
10	Detection wavelength	214nm
11	Injection volume	5µl
12	Retention time (RT)	3.423 ± 0.400 min
13	Run time	6.00 min

Method Validation:

The method was validated in compliance with ICH guidelines. The parameters determined for validation were specificity, precision, accuracy, robustness, linearity, Limit of Quantification and Limit of Detection, system suitability and stability of analytical solution.

Specificity:
The method specificity was assessed by comparing the chromatograms obtained from a placebo solution containing a mixture of most commonly used excipients without the drug and another solution containing the excipients with the drug. These solutions were prepared in the diluent. The drug to excipient ratio used was similar to that in the commercial formulation. The mixtures were filtered through 2µ membrane filter before injection. The placebo solution and the sample solution (placebo and the drug) were injected into HPLC system separately in triplicate and the relevant chromatograms observed.

Precision:
System precision:
Six replicates of standard solution of Zopiclone were injected into HPLC system.

Method precision:
The precision of the procedure was determined by repeatability. Six sample preparations were made from a single batch of Zopiclone tablets and analyzed as per the proposed method.

Intermediate precision (Ruggedness):
Ruggedness of method was verified by analyzing six sample preparations of same batch used under method precision as per proposed method by different analysts using different instrument and on different day. The amount of Zopiclone in Zopiclone tablets was determined. % RSD for % assay of Zopiclone was calculated, for six preparations.

Accuracy:
The placebo was spiked with known amounts of Zopiclone (API) at about 50%, 100% and 150% of test concentration prepared in triplicate at each level. Amount of Zopiclone was quantified and % recovery was calculated from amount found and actual amount added. % Recovery at each level was calculated.

Linearity:
Linearity of response was performed using the standard solution in a range of 50ppm to 300ppm [50% - 150% of the test concentration].

Stability in analytical solution:
Stability of Zopiclone in analytical solution was verified by analyzing sample solution initially and also at different time intervals up to 48 hrs when the sample was stored at room temperature.

Robustness:
To evaluate robustness, following variations were made in the method and the samples were analyzed in triplicate. Change in Flow rate by (10%), Change in Organic content variation in mobile phase (± 2mM). System suitability was evaluated in each condition and results were compared with method precision results.

Limit of Detection and Limit of Quantification:
Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a measurable response. LOD is determined based on signal to noise ratio (S/N) of three times typically for HPLC methods. The limit of quantification (LOQ) is defined as the lowest concentration that can be quantified reliably with a specified level of accuracy and precision.

RESULTS AND DISCUSSION: A mixture of mobile phase composed of ammonium bicarbonate (5 mM, neutral) and acetonitrile in a gradient mode at a flow rate of 1 ml/min was found to be a suitable solvent system. **Fig. 2** shows a typical chromatogram obtained from the analysis of a reference standard using the proposed method for zopiclone. As shown in this figure, a symmetrical peak represents zopiclone. The retention time observed in the assay (3.28 min) associated with the simple sample preparation (for tablets) allowed a rapid determination of the drug in pharmaceutical products **Fig.3**.

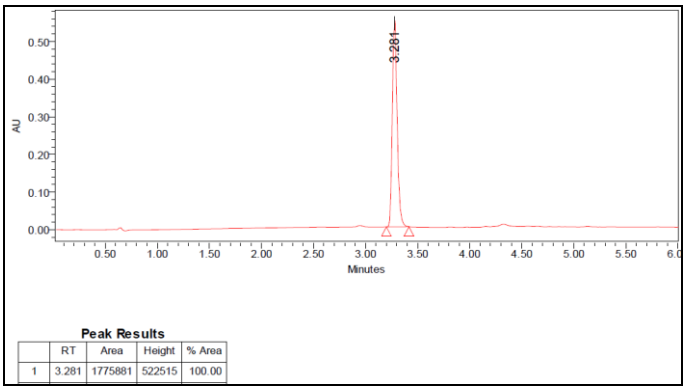

FIG. 2: A REPRESENTATIVE CHROMATOGRAM OF STANDARD SOLUTION

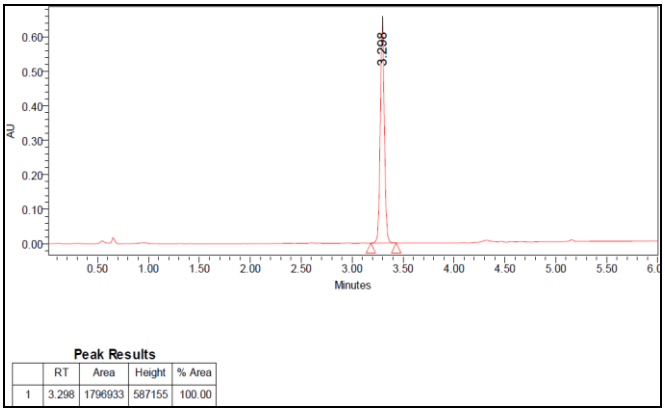

FIG. 3: A REPRESENTATIVE CHROMATOGRAM OF SAMPLE SOLUTION

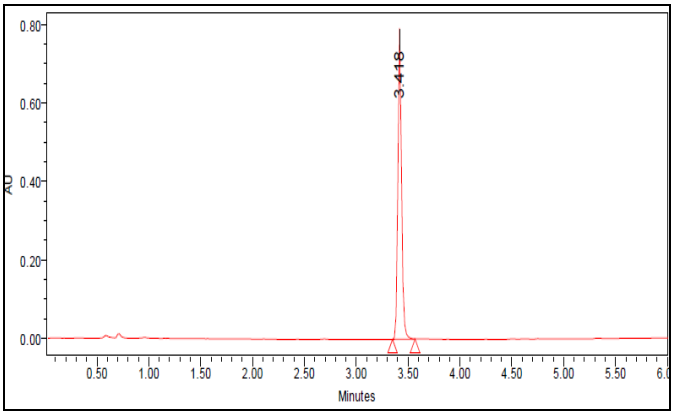

FIG. 4: A REPRESENTATIVE CHROMATOGRAM OF ZOPICLONE FROM THE TABLET SOLUTION

Specificity:
There was no interference from blank and placebo at the retention time of analyte peak. The absence of additional peaks in the chromatogram indicates non interference of the commonly used excipients in the tablets and hence the method is specific **Fig. 4**.

Precision:
System Precision: % RSD of Zopiclone peak area counts from six replicate injections of standard solution was less than 2.0 and it meets the acceptance criterion. The results were summarized in **Table 2**.

TABLE 2: SYSTEM PRECISION DATA

S. No.	Replicate	Standard Area	Sample	Sample Area
1	Replicate-1	2059527		
2	Replicate-2	2068670	Sample 1	2151964
3	Replicate-3	2066117		
4	Replicate-4	2066515		
5	Replicate-5	2081561	Sample 2	2151982
6	Replicate-6	2089489		
	Average	2071979.8	**2151973**	
	SD	11212.80	**12.73**	
	%RSD	0.54	**0.00**	
%Assay 102.8%				

Method precision:
The %RSD for %Assay of Zopiclone for six sample preparations was less than 2.0% and meets the acceptance criterion.

Intermediate precision (Ruggedness): % RSD for % assay of Zopiclone was less than 2.0% and meets the acceptance criteria. Overall % RSD for % assay of Zopiclone obtained from ruggedness and method precision was less than 2.0% and meets the acceptance criteria. The results were summarized in **Table 3.**

TABLE 3: PRECISION DATA WITH RUGGEDNESS

S. No.	Sample	% Assay
1	Precision 1	100.2
2	Precision 2	100.5
3	Precision 3	100.7
4	Precision 4	100.8
5	Precision 5	100.7
6	Precision 6	100.7
7	Robustness 1	101.6
8	Robustness 2	100
9	Robustness 3	100.4
10	Robustness 4	103.5
	Average	**100.91**
	SD	**1.004**
	% RSD	**0.99**

Accuracy:
Analytical method meets acceptance criteria for recovery study. Hence the method is accurate and precise. The results were summarized in **Table 4**.

Linearity: The results are tabulated in **Table 5** and represented graphically **Fig. 5**. The relevant correlation coefficient value is more than 0.99.

TABLE 4: ACCURACY DATA

Spike Level	Sample area	Average area	Sample Wt. (mg)	Amount added (µg)	Amount recovered (µg)	% Recovery	Average % recovery	SD	% RSD
50%	1004636	1006953	25	250	258.81	100.52	103.76	0.23	0.22
	1009150				259.97	100.99			
	1007073				259.44	100.77			
100%	2004082	1999895.8	25	500	513.06	102.61	102.76	0.34	0.33
	1991575				515.54	103.11			
	2009917				516.28	103.26			
150%	2932132	2942735	25	750	755.36	100.71	101.08	0.33	0.33
	2944956				758.66	101.16			
	2951118				760.25	101.37			

TABLE 5: LINEARITY DATA

S. No.	RT(min)	STD Area
Replicate-1	3.462	1678248
Replicate-2	3.462	1616277
Replicate-3	3.461	1623211
Replicate-4	3.462	1656613
Replicate-5	3.462	1646851
Replicate-6	3.462	1627188
Mean	3.461	1641398
SD	0.00	23591.99
%RSD	**0.00**	**1.43**

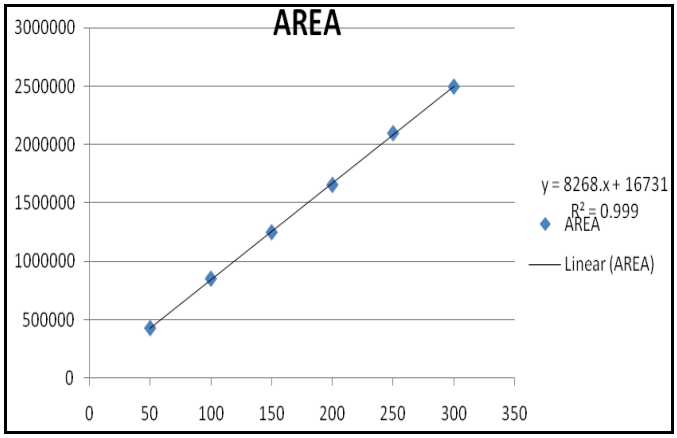

FIG. 5: LINERAITY PLOT

Stability in analytical solution:

Cumulative %RSD is less than 2.0 at each time interval. The results are tabulated in **Table 6**.

TABLE 6: SOLUTION STABILITY DATA

Hours	RT	Area	% Assay
0 Hr	3.666	2124908	96.7
12 Hr	3.664	2166315	98.6
24 Hr	3.667	2193228	99.9
48 Hr	3.628	2196776	100.0
Average	**3.656**	**2170306**	**98.8**
SD	**0.018**	**33180.79**	**1.53**
%RSD	**0.49**	**1.52**	**1.54**

The solution was found to be stable up to 48 hrs at room temperature. Hence it is concluded that the proposed analytical method meets the pre-established acceptance criteria.

Robustness:

The results obtained are tabulated in **Table 7** and **8**. Overall %RSD is than 2.0% for individual experiment. As method meets pre-established acceptance criterion, the method is considered to be robust for small changes in flow rate, concentration of buffer for mobile phase.

TABLE 7: CHANGE IN FLOW RATE DATA

a) For increase in flow

Replicate	Standard area	Sample area	% Assay
Replicate-1	1745476	1771424	**100.4**
Replicate-2	1764112		
Replicate-3	1756160		
Replicate-4	1736315	1775200	
Replicate-5	1743366		
Replicate-6	17480170		
Average	**1748907.7**	**1773312**	
SD	**9855.18**	**2670.03**	
%RSD	**0.56**	**0.150**	

b) For decrease in flow

Replicate	Standard Area	Sample Area	% Assay
Replicate-1	2092695	2205052	**103.5**
Replicate-2	2097550		
Replicate-3	2110513		
Replicate-4	2093387	2202254	
Replicate-5	2122197		
Replicate-6	2093387		
Average	**2108581.3**	**2203653**	
SD	**7931.44**	**1978.48**	
%RSD	**0.38**	**0.09**	

TABLE 8: CHANGE IN BUFFER CONCENTRATION DATA

a) Increase in buffer concentration:

Replicate	Standard area	Sample area	% Assay
Replicate-1	1860346		
Replicate-2	1854263	1909252	
Replicate-3	1861863		
Replicate-4	1854924		
Replicate-5	1854534	1902761	**101.6**
Replicate-6	1852915		
Average	**185674.2**	**1906006.5**	
SD	**3681.16**	**4589.83**	
%RSD	**0.20**	**0.24**	

b) Decrease in buffer concentration

Replicate	Standard area	Sample area	% Assay
Replicate-1	1916050		
Replicate-2	1960701	1924832	
Replicate-3	1888299		
Replicate-4	1909604		
Replicate-5	1896047	1913332	**100**
Replicate-6	1884475		
Average	**1900196**	**1919082**	
SD	**12554.50**	**8131.73**	
%RSD	**0.66**	**0.42**	

Limit of Detection and Limit of Quantification:

The LOD and LOQ of Zopiclone obtained by the proposed method were 9.416 and 28.534µg/mL respectively.

CONCLUSION: Developed assay method is simple, rapid, accurate, precise, economical, specific and reproducible for the quantitative determination of Zopiclone with good resolution in short time and high sensitivity.

The flow rate programming of gradient mobile phase, cut down an overall time of sample analysis and thereby made the method more cost effective and rapid and produced a quicker method for zopiclone detection using basic buffer.

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