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Development and Validation of High-Performance Liquid Chromatographic Method for Analysis of Zolpidem in Marketed Sublingual Spray

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Abstract: *A rapid, sensitive, and reliable High-Performance Liquid Chromatographic (HPLC) method was developed and validated for the quantitative determination of Zolpidem tartrate (ZOL), a widely used sedative and hypnotic. The chromatographic separation was achieved on a reversed-phase C18 column using an isocratic mobile phase consisting of 0.1% OPA and Methanol (50:50 v/v) at a flow rate of 1.0 mL/min. UV detection was carried out at 293 nm. The method was validated according to USP guidelines for linearity, range, accuracy, precision (repeatability and intermediate precision), specificity (placebo interference), and robustness. The method exhibited good linearity over the concentration range of 1.2-2.7 µg/mL with a correlation coefficient (R^2) of 0.999. Accuracy, determined by recovery studies, was within the acceptable limits of 98-102%. Precision, expressed as the relative standard deviation (%RSD), was less than 2.0% for both repeatability (0.45%) and intermediate precision (0.82%). The method was specific, with no interference from placebo. Robustness was demonstrated by evaluating the effect of small deliberate changes in flow rate, organic phase composition, and wavelength. The developed and validated HPLC method is suitable for routine quality control analysis of Zolpidem in pharmaceutical sublingual spray formulations.*

Keywords: Zolpidem tartrate, HPLC, Method Development, Validation, Pharmaceutical Analysis, Quality Control

I. INTRODUCTION

Zolpidem tartrate(N,N-dimethyl-2-[6-methyl-2-(4-methyl phenyl)imidazo[1,2-a]pyridin-3-yl]acetamide) is a sedative-hypnotic medication that acts as a positive allosteric modulator at the GABA receptor, enhancing the inhibitory effects of the neurotransmitter GABA. Zolpidem widely used for insomnia treatment. Accurate and reliable analytical methods are crucial for the quality control of Zolpidem tartrate in pharmaceutical formulations. High-Performance Liquid Chromatography (HPLC) is a preferred technique for such analysis due to its sensitivity and selectivity (9).While some analytical methods for ZOL estimation have been reported, there is a continuous need for simple, sensitive, and validated methods for routine analysis. This study aimed to develop and validate a simple RP-HPLC method for the quantitative determination of Zolpidem tartrate in sublingual spray formulations, following USP guidelines.

II. MATERIALS AND METHODS

2.1. Materials

Zolpidem tartrate reference standard (purity 99.9% w/w) was obtained from Arrow Chem Mumbai (Table No. 1).

Drug	Supplied by	Quantity	Purity (Assay)
Zolpidem Tartrate	Arrow Chem Mumbai.	10 g	99.9 % w/w

Table No. 1: Details of API

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Ataxin® 50 mg tablets (SavaVet Pharma Ltd) were obtained from the local market (Table No. 2).

Brand Name	Mfd by	Content	Quantity
Zolswift-SL® Spray	Troikaa Pharma	Zolpidem Tartrate 3.85% w/v	3.85% w/v

Table No. 2: Details of marketed Preparation

- HPLC grade Methanol and Water were used.
- HPLC grade Ortho Phosphoric Acid was used.
- The placebo composition of Zolswift-SL® Spray was used for the placebo interference study.

2.2. Instruments

Table No. 3: Instruments Used

Sr.No	Instruments	Make	Model
1	UV-Visible Spectrophotometer	Shimadzu	UV 1900i
2	HPLC	Waters 600	996 PDA Detector
3	pH Meter	Hanna	-
4	Balance	Citizen	CY 104 (Micro Analytical Balance)
5	Ultra sonicator	-	1.5 L 50

- HPLC system equipped with a Waters 600 pump, a Waters 996 PDA Detector, and Empower software for data acquisition (Table No. 10).
- Analytical balance with a sensitivity of 0.1 mg (Citizen CY 104 Micro Analytical Balance).
    - pH meter (Hanna).
    - Ultrasonicator (1.5 L 50).
    - UV-Visible Spectrophotometer (Shimadzu UV 1900i) was used for determining the wavelength maxima.
    - HPLC column: C18 (Thermo Hypersil gold), 5 µm particle size, 250 mm x 4.6 mm.

2.3. Chromatographic Conditions

- Mobile Phase: 0.1% OPA:Methanol(50:50 v/v). The 0.1% OPA was prepared by dissolving 1 mL of Orthophosphoric acid in 1000 mL of HPLC grade water and mix well.
- Flow Rate: 1.0 mL/min.
- Detection Wavelength: 293nm (Fig. No. 1).
- Column temperature: Ambient (25°C)

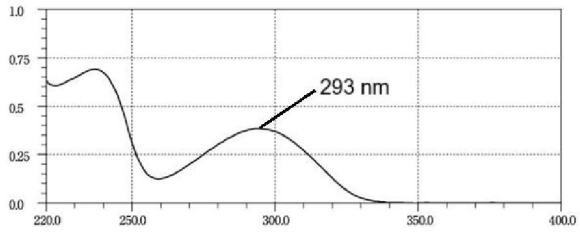

Fig. No. 1: Wavelength Maxima Zolpidem tartrate

- Injection Volume: 20 µL.
- Run Time: 20 minutes.

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2.4. Preparation of Standard Solutions

- A stock standard solution of Zolpidem tartrate (150µg/mL) was prepared by dissolving 15 mg of the reference standard in Methanol in a 100 mL volumetric flask. For method development, a 150 µg/mL solution was also prepared.
- Working standard solutions (1.5 µg/mL) for system suitability, accuracy, and method precision studies were prepared by diluting the stock standard solution with Methanol.
- Working standard solutions for linearity studies were prepared at concentrations of 1.2, 1.5,1.8, 2.4, and 2.7 µg/mL by appropriate dilutions of the stock standard solution with Acetonitrile.

2.5. Preparation of Sample Solutions

- Entire content of Zolswift-SL (3.85% w/v) spray (3.9 ml) was transferred to a 500 ml volumetric flask, the volume was made upto the mark with methanol, the resultant concentration was 300 µg/ml. The whole content was centrifuged at 5000 rpm for 10 min followed by passing through 0.45 µ membrane filter. 1 ml of resultant was transferred to a 200 ml volumetric flask and the volume was made upto the mark with methanol, the concentration of working sample solution was 1.50 µg/ml.

2.6. Method Validation

The developed HPLC method was validated according to USP guidelines.

2.6.1. Specificity

2.6.1.1 System suitability test : Filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 mL std. drug solution was injected which was made in five replicates and the system suitability parameters were recorded.

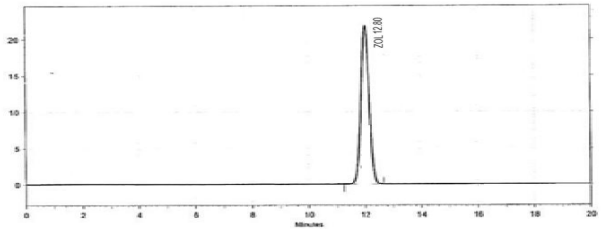

Fig. No. 2: Chromatograms of system suitability test

Sr.No.	Peak area	Retention Time	Symmetry	No. of theoretical Plates
	ZOL	ZOL	ZOL	ZOL
1	150269	12.12	1.20	9526
2	148952	12.20	1.10	9588
3	145896	12.05	1.25	9645
4	152698	12.09	1.00	9600
5	147895	12.08	1.20	9550
Mean	149142	12.108	1.15	9582
S.D	551.61	0.057	0.1	46.07
%R.S.D	0.34	0.47	1.5	0.48

Table No. 4: Result of System suitability test

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Brand name : Zolswift-SL® sublingual spray

Sr.no.	ZOL	
	Assay (mg)	Assay % of LC
1	1.49	99.33
2	1.50	100.00
3	1.50	100.00
4	1.49	99.33
5	1.49	99.33
Average	1.494	99.60
SD	0.005	0.36
% RSD	0.36	0.36

Table No. 5: Results and statistical data for estimation of ZOL in marketed formulation

2.6.1.2 Placebo Interference Study

A placebo solution was prepared following the same procedure as the sample preparation but without the active ingredient. The chromatogram of the placebo solution was compared with that of the standard to check for any interference at the retention time of Zolpidem tartrate (Fig. No. 3, Table No. 6).

Placebo Preparation

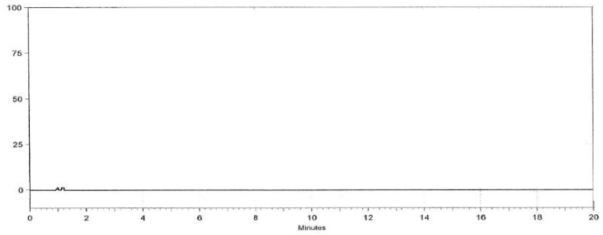

Fig. No. 3: Chromatograms of placebo interference study

Observation	Placebo prep.1	Placebo prep.2	Placebo prep.3
% Interference	No Interference	No Interference	No Interference

Table No. 6: Placebo Interference

2.6.2. Linearity and Range

Linearity was evaluated by injecting standard solutions at five concentration levels (1.2, 1.5, 1.8, 2.4, and 2.7 µg/mL) in triplicate. The peak area was plotted against the concentration, and the correlation coefficient (R^2) was calculated (Fig. No. 4, Table No.7).

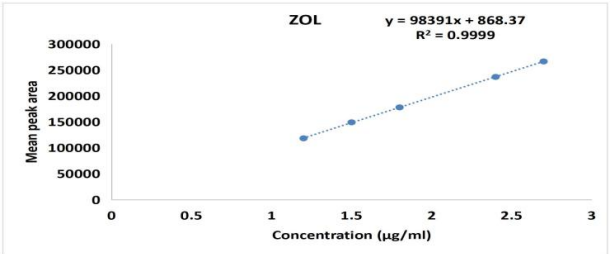

Fig. No. 4: Plot of linearity and range study for ZOL

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Sr. No.	% Level	ZOL	
		Conc. (µg/ml)	Mean peak
1	80	1.2	118360
2	100	1.5	149200
3	120	1.8	178040
4	160	2.4	236720
5	180	2.7	266580

Table No. 7 : Observations of Linearity and range study for ZOL

2.6.3. Accuracy

Accuracy was determined by recovery studies using the standard addition method. Known amounts of Zolpidem tartrate standard (1.2, 1.5, and 1.8 µg/mL) were spiked into a placebo. Each spiking level was prepared in triplicate, and the percentage recovery was calculated (Table No. 8).

	ZOL		
	Levels		
	80%	100%	120%
Amt added (µg/ml)	1.2	1.5	1.8
	1.2	1.5	1.8
	1.2	1.5	1.8
Amt taken (µg/ml)	1.2	1.5	1.8
	1.2	1.5	1.8
	1.2	1.5	1.8
Amt recovered (µg/ml)	1.18	1.49	1.79
	1.18	1.48	1.78
	1.19	1.49	1.80
%Recovery	98.33	99.33	99.44
	98.33	98.66	98.88
	99.16	99.33	100.00
Mean % recovery	98.60	99.10	99.44
% RSD	0.48	0.39	0.56

Table No. 8: Accuracy studies by standard addition method

2.6.4. Precision:

System Precision: The standard working solution was injected five times, and the %RSD of the peak areas and retention times was calculated (Table No.9, Fig. No. 5)

Sr. No.	Parameter	Observations	Limits
1	The % RSD of peak area response for three replicate injections of standard	1.217	NMT 2.0
2	Theoretical plates	8057.53	NLT 2000
3	Tailing factor	1.278	NMT 2.0

Table No. 9: Results for System Precision showing system suitability

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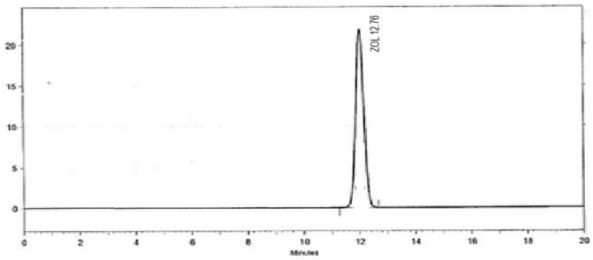

Fig. No. 5: Chromatogram System precision Showing Repeatability

Method Precision (Repeatability): Three independent sample preparations were analyzed, and the %RSD of the assay results was calculated (Fig. No. 6, Table No.10)

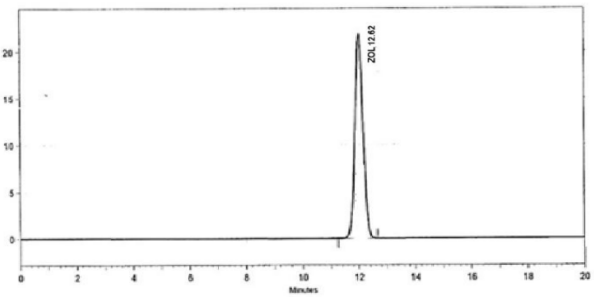

Fig. No. 6: Chromatogram of Method precision

Sr.no.	ZOL	
	Assay (mg)	Assay % of LC
1	1.501	100.1
2	1.492	99.5
3	1.506	100.4
Average	1.499	100
SD	0.70	0.45
% RSD	0.47	0.45

Table No. 10: Method Precision Studies Set – I

Intermediate Precision (Ruggedness): Three sample preparations were analyzed on a different HPLC system, using a different column, and by a different analyst on a different day. The %RSD of the assay results was calculated and compared with the method precision (Table No. 11, Table No. 12).

Sr.No.	ZOL	
	Assay (mg)	Assay % of LC
1	1.494	99.6
2	1.512	100.8
3	1.488	99.22
Average	1.498	99.87
SD	0.012	0.82
% RSD	0.83	0.82

Table No. 11: Intermediate precision Studies (Ruggedness) Set – II

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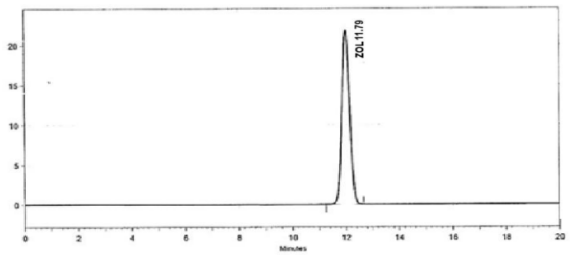
Sr.no.	% Assay of LC	
	ZOL	
	Set – I	Set - II
1	100.1	99.6
2	99.5	100.8
3	100.4	99.22
Average	100.2	
SD	0.83	
% RSD	0.83	

Table No. 12: Intermediate precision (Ruggedness) evaluation of data

2.6.5. Robustness

The effect of small deliberate changes in flow rate (0.9 and 1.1 mL/min), mobile phase composition (55:45 and 45:55 Phosphate buffer:Acetonitrile), and detection wavelength (275 and 279 nm) on system suitability parameters was evaluated (Fig. No.7, Fig. No. 8, Fig. No. 9,Table No. 13, Table No. 14,Table No. 15).

0.9 ml/min



1.1 ml/min

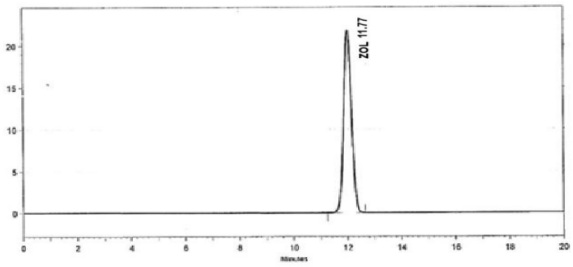
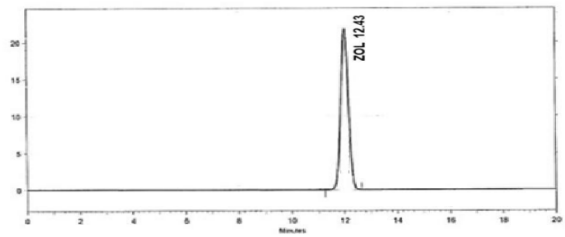


Fig. No. 7: Chromatograms of Change in Flow Rate

-10% ACN:- (Phosphate buffer pH 3.5: ACN 55:45)



+10% ACN: (Phosphate buffer pH 3.5: ACN 45:55)

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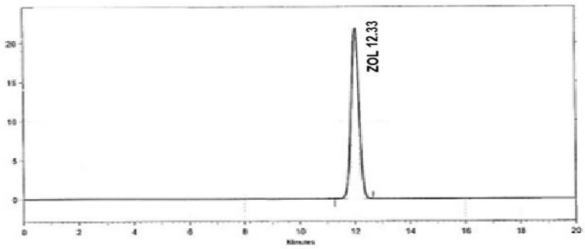
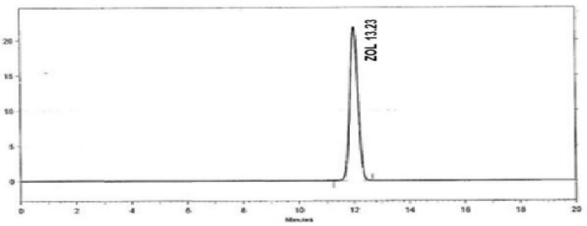


Fig. No. 8: Chromatograms of Change Organic Composition of mobile Phase
At 291nm wavelength



At 295nm wavelength

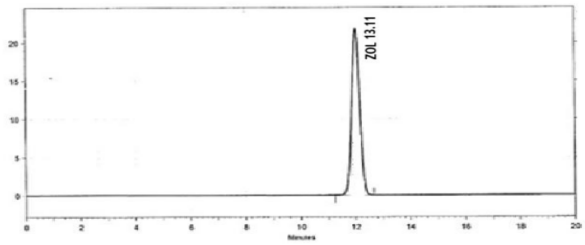


Fig. No. 9: Chromatograms of Change Organic Composition of mobile Phase

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	0.9mL	1.1 mL	
1	The % RSD of peak area response for five replicate injections	ZOL	1.027	0.92	0.85	NMT 2.0
2	Theoretical plates	ZOL	7197.53	7138.7	7557.9	NLT 2000
3	Tailing factor	ZOL	1.28	1.91	1.10	NMT 2.0
4	Retention Time (Min)	ZOL	11.81	11.79	11.77	

Table No.13: System suitability of change in flow rate

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	- 10%	+ 10%	
1	The % RSD of peak area response for five replicate injections	ZOL	1.017	0.655	0.046	NMT 2.0
2	Theoretical plates	ZOL	7197.53	7996	6347.6	NLT 2000
3	Tailing factor	ZOL	1.28	1.166	1.08	NMT 2.0
4	Retention Time (Min)	ZOL	11.69	12.43	12.33	

Table No.14: System suitability of change in mobile phase composition

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Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	291 nm	295 nm	
1	The % RSD of peak area response for five replicate injections	ZOL	1.017	0.3638	0.141	NMT 2.0
2	Theoretical plates	ZOL	8057.53	7987.9	6678.3	NLT 2000
3	Tailing factor	ZOL	1.06	1.00	0.94	NMT 2.0
4	Retention Time (Min)	ZOL	12.85	13.23	13.11	

Table No.15: System suitability of change in wavelength

III. RESULTS AND DISCUSSION

The developed RP-HPLC method provided a good separation of Zolpidem tartrate with a retention time of approximately 12.80 minutes (Fig. No.10). The system suitability parameters were within acceptable limits (Table No.15), indicating the proper functioning of the chromatographic system.

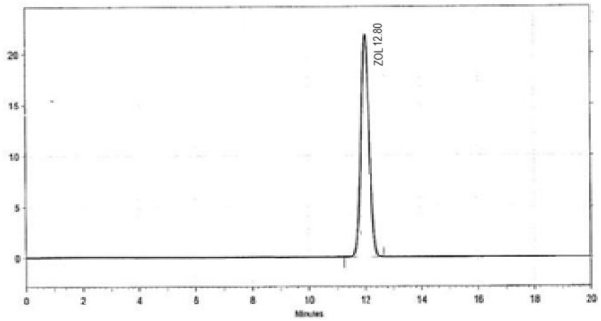

Fig. No. 10: Separation of ZOL in selected mobile phase showing retention time at 12.80 min

The method validation results demonstrated that the method is specific, as no interference was observed from the placebo at the retention time of Zolpidem tartrate (Fig. No.11, Table No. 16)

Placebo Preparation

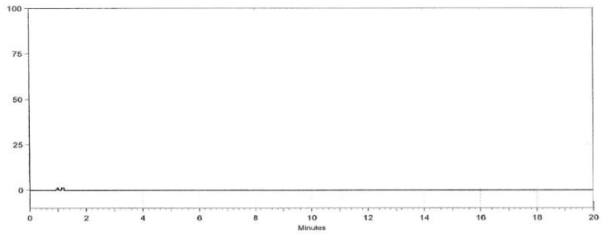

Fig. No. 11: Chromatograms of placebo interference study

Observation	Placebo prep.1	Placebo prep.2	Placebo prep.3
% Interference	No Interference	No Interference	No Interference

Table No.16: Placebo Interference

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Sr.No.	Peak area	Retention Time	Symmetry	No. of theoretical Plates
	ZOL	ZOL	ZOL	ZOL
1	150269	12.12	1.20	9526
2	148952	12.20	1.10	9588
3	145896	12.05	1.25	9645
4	152698	12.09	1.00	9600
5	147895	12.08	1.20	9550
Mean	149142	12.108	1.15	9582
S.D	551.61	0.057	0.1	46.07
%R.S.D	0.34	0.47	1.5	0.48

Table No.17: Result of System suitability test

The method exhibited good linearity in the concentration range of 40-90 µg/mL with a correlation coefficient of 0.999 (Fig. No. 12, Table No. 18).

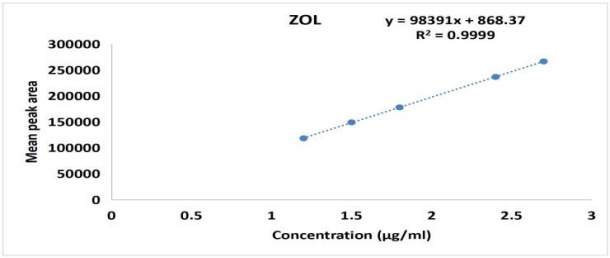

Fig. No. 12: Plot of linearity and range study for ZOL

Sr. No.	% Level	ZOL	
		Conc.	Mean peak area
1	80	1.2	118360
2	100	1.5	149200
3	120	1.8	178040
4	160	2.4	236720
5	180	2.7	266580

Table No.18: Observations of Linearity and range study for ZOL

The accuracy of the method, assessed by recovery studies, was within the acceptable range of 98-102% (Table No. 19)

	ZOL		
	Levels		
	80%	100%	120%
Amt added (µg/ml)	1.2	1.5	1.8
	1.2	1.5	1.8
	1.2	1.5	1.8
Amt taken (µg/ml)	1.2	1.5	1.8
	1.2	1.5	1.8
	1.2	1.5	1.8
Amt	1.18	1.49	1.79

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recovered (µg/ml)	1.18	1.48	1.78
	1.19	1.49	1.80
% Recovery	98.33	99.33	99.44
	98.33	98.66	98.88
	99.16	99.33	100.00
Mean % recovery	98.60	99.10	99.44
% RSD	0.48	0.39	0.56

Table No 19: Accuracy studies by standard addition method

The method showed good precision, with %RSD values for system precision (Table No.20)

Sr. No.	Parameter	Observations	Limits
1	The % RSD of peak area response for three replicate injections of standard	1.217	NMT 2.0
2	Theoretical plates	8057.53	NLT 2000
3	Tailing factor	1.278	NMT 2.0

Table No.20: Data showing system Precision

and method precision (Table No. 21) being less than 2.0%. Intermediate precision studies also yielded acceptable %RSD values (Table No.22, Table No. 23)

Sr.no.	ZOL	
	Assay (mg)	Assay (mg)
1	1.501	100.1
2	1.492	99.5
3	1.506	100.4
Average	1.499	100
SD	0.70	0.45
% RSD	0.47	0.45

Table No.21: Method Precision Studies Set – I

Sr.No.	ZOL	
	Assay (mg)	Assay % of LC
1	1.494	99.6
2	1.512	100.8
3	1.488	99.2
Average	1.498	99.87
SD	0.012	0.82
% RSD	0.83	0.82

Table No.22: Intermediate precision Studies (Ruggedness) Set – II

Sr.no.	% Assay of LC	
	ZOL	
	Set – I	Set - II
1	100.1	99.6
2	99.5	100.8

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3	100.4	99.2
Average	100.2	
SD	0.83	
% RSD	0.83	

Table No.23: Intermediate precision (Ruggedness) evaluation of data

indicating the ruggedness of the method. The robustness of the method was confirmed as small changes in chromatographic conditions did not significantly affect the system suitability parameters (Table No. 24, Table No. 25, Table No. 26).

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	- 10%	+ 10%	
1	The % RSD of peak area response for five replicate injections	ZOL	1.017	0.655	0.046	NMT 2.0
2	Theoretical plates	ZOL	7197.53	7996	6347.6	NLT 2000
3	Tailing factor	ZOL	1.28	1.166	1.08	NMT 2.0
4	Retention Time (Min)	ZOL	11.69	12.43	12.33	

Table No. 24: System suitability of change in Organic Composition

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	275 nm	279 nm	
1	The % RSD of peak area response for five replicate injections	ZOL	1.017	0.3638	0.141	NMT 2.0
2	Theoretical plates	ZOL	8057.53	7987.9	6678.3	NLT 2000
3	Tailing factor	ZOL	1.06	1.00	0.94	NMT 2.0
4	Retention Time (Min)	ZOL	12.85	13.23	13.11	

Table No.25: System suitability of change in wavelength

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	0.9mL	1.1 mL	
1	The % RSD of peak area response for five replicate injections	ZOL	1.027	0.92	0.85	NMT 2.0
2	Theoretical plates	ZOL	7197.53	7138.7	7557.9	NLT 2000
3	Tailing factor	ZOL	1.28	1.91	1.10	NMT 2.0
4	Retention Time (Min)	ZOL	11.81	11.79	11.77	

Table No.26: System suitability of change in Flow rate

IV. SUMMARY AND CONCLUSION

4.1. Summary

Sublingual spray formulation containing ZOL is recently introduced in market to treat insomnia condition. Literature survey revealed very few methods for the estimation of ZOL.The present study was undertaken with an objective of developing suitable, sensitive and simple analytical RP-HPLC method for estimation of ZOL in the sublingual spray formulation.In the developed RP-HPLC method the analyte were resolved using Mobile phase composed of water (0.1% OPA) and methanol in the ratio 50:50 % v/v. A isocratic program was developed contributinga total run time of 10 min. using HPLC auto-sampler system containing PDA detector with EMPOWER Software and C_{18} (Thermo Hypersil gold) /4.6 x 250 mm column, the detection wavelength was 293 nm. The method gave the good resolution

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and suitable retention time.The results of analysis in all the method were validated in terms of accuracy, precision, ruggedness, linearity and range. The methods were found to be sensitive, reliable, reproducible, rapid and economic also.

4.2. Conclusion

From the results of the study it can be concluded that the present RP-HPLC technique was successfully used for the estimation of the ZOL in the sublingual spray formulation.The method showed good reproducibility, it was accurate, precise, specific, reproducible and sensitive. The analysis of Sublingual spray formulation of ZOL was done by the developed and validated RP-HPLC method.The RP-HPLC method was also simple, accurate, precise, reproducible and economical too. It may be adopted for routine control analysis of ZOL alone and in combined dosage form.No interference of additives, matrix etc. is encountered in these methods. Further studies on other pharmaceutical formulations would throw more light on these studies.Suitability of these methods on biological samples needs to be studed.

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