

A new analytical method development and validation for the simultaneous estimation of melatonin and zolpidem tartarate using RP-HPLC

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ABSTRACT

A simple and selective LC method is described for the determination of Melatonin and Zolpidem tartarate in tablet dosage forms. Chromatographic separation was achieved on a C_{18} column using mobile phase consisting of a mixture of 55 volumes of water and 45 volumes of Methanol with detection of 280nm. Linearity was observed in the range 20-100 $\mu\text{g/ml}$ for Melatonin ($r^2 = 0.999$) and 20-100 $\mu\text{g/ml}$ for Zolpidem tartarate ($r^2 = 0.999$) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.

The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

INTRODUCTION

A drug includes all medicines intended for internal or external use for or in the diagnosis, treatment, mitigation or prevention of disease or disorder in human beings or animals, and manufactured exclusively in accordance with the formulae mentioned in authoritative books [1-3].

Pharmaceutical analysis is a branch of chemistry involving a process of identification [4-6], determination, quantification, purification and separation of components in a mixture or determination of chemical structure of compounds [7-10]. There are two main types of analysis – Qualitative and Quantitative analysis.

AIM AND PLAN OF WORK

Aim

To develop new RP HPLC method for the simultaneous estimation of MELATONIN and ZOLPIDEM TARTARATE pharmaceutical dosage form.

Plan of work

- Solubility determination of MELATONIN and ZOLPIDEM TARTARATE various solvents and buffers.
- Determine the absorption maxima of both the drugs in UV-Visible region in different solvents/buffers and selecting the solvents for HPLC method development.

- Optimize the mobile phase and flow rates for proper resolution and retention times.
- Validate the developed method as per ICH guidelines.

METHODOLOGY

Mobile Phase

A mixture of 80 volumes of Methanol and 20 volumes of Water. The mobile phase was sonicated for 10min to remove gases.

Determination of Working Wavelength (λ_{max})

In estimation of drug wavelength maxima is used.. So this wavelength is used in estimation to estimate drug accurately.

Preparation of standard stock solution of MELATONIN

10 mg of ZOLPIDEM TARTARATE was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 μg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of standard stock solution of ZOLPIDEM TARTARATE

10 mg of MELATONIN was weighed in to 100ml volumetric flask and dissolved in Methanol and then dilute up to the mark with methanol and prepare 10 μg /ml of solution by diluting 1ml to 10ml with methanol.

RESULTS AND DISCUSSIONS

Solubility studies

These studies are carried out at 25 $^{\circ}\text{C}$

Melatonin

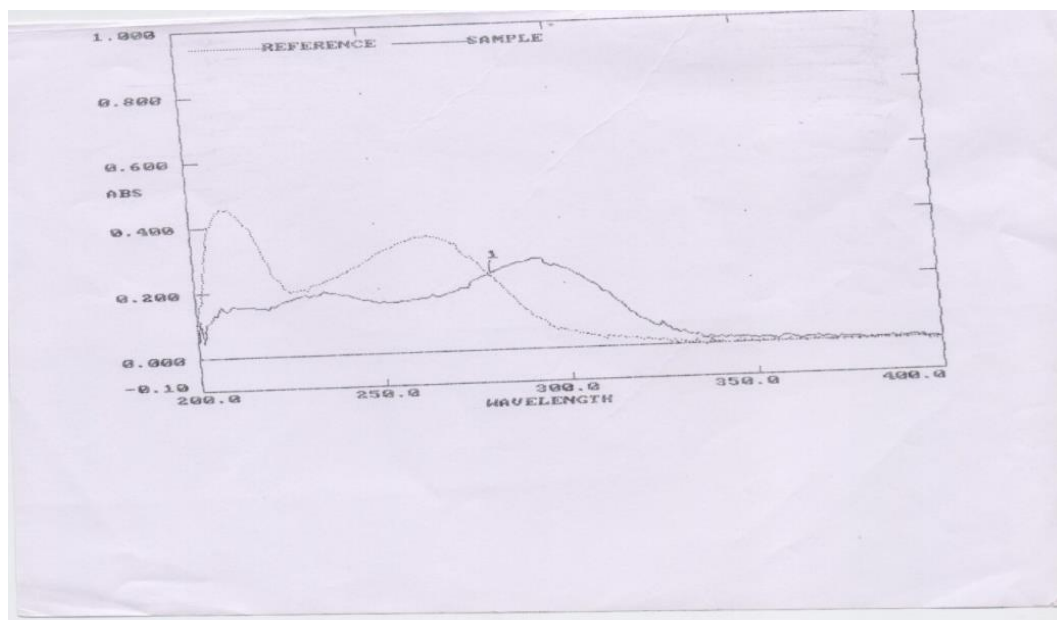
Freely soluble in methanol, water and mixed phosphate buffer.

Zolpidem tartarate

Freely soluble in ethanol and methanol, and slightly soluble in acetone and isopropanol and very slightly soluble in water

Wavelength determination

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.



UV-VIS spectrum of ZOLPIDEM TARTARATE and MELATONIN and the isosbestic point was 280 nm

Observation

The Isobestic point was found to be 280 nm for ZOLPIDEM TARTARATE and MELATONIN in combination

METHOD DEVELOPMENT OF ZOLPIDEM TARTARATE AND MELATONIN

Trial- 1

Preparation of standard solution

Weigh accurately 10 mg of ZOLPIDEM TARTARATE and MELATONIN in 100 ml of

volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μ g/ml of ZOLPIDEM TARTARATE and MELATONIN is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

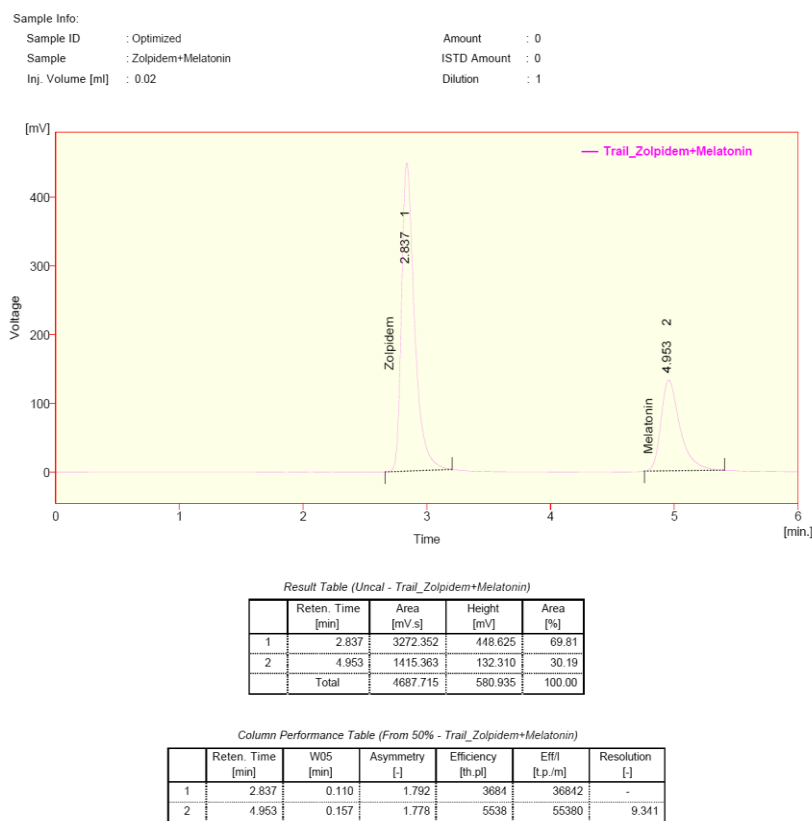


Fig. 3: Chromatogram of ZOLPIDEM TARTARATE AND MELATONIN

Observation

- Peak Asymmetry factor for ZOLPIDEM TARTARATE and MELATONIN meet the system suitability requirements.

- The run time is very correct.
- Theoretical plates were more than 2000. Hence it is taken for optimization.

Table 1: Optimized chromatographic conditions

Mobile phase	Methanol: Water
pH	-
Column	Inertsil ODS 3V column,C18(150x4.6 ID) 5µm
Flow rate	1.0 ml/min
Column temperature	Room temperature(20-25°C)
Sample temperature	Room temperature(20-25°C)
Wavelength	280
Injection volume	20 µl
Run time	6 min
Retention time	About 4.953 min for ZOLPIDEM TARTARATE and 2.837 min for MELATONIN.

ASSAY

Preparation of samples for Assay

Preparation of standard solution

Preparation of mixed standard solution

Weigh accurately 10mg of ZOLPIDEM TARTARATE and 10 mg of MELATONIN in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of ZOLPIDEM TARTARATE and MELATONIN is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains MELATONIN-10 mg, ZOLPIDEM TARTARATE-20 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of MELATONIN and ZOLPIDEM TARTARATE (µg/ml) were prepared by dissolving weight equivalent to 10 mg of MELATONIN and ZOLPIDEM TARTARATE and dissolved in

sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are prepared in 5 replicates of 10µg/ml of MELATONIN and ZOLPIDEM TARTARATE was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Calculation

The amount of MELATONIN and ZOLPIDEM TARTARATE present in the formulation by using the formula given below, and results shown in above table:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of MELATONIN /ZOLPIDEM TARTARATE in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

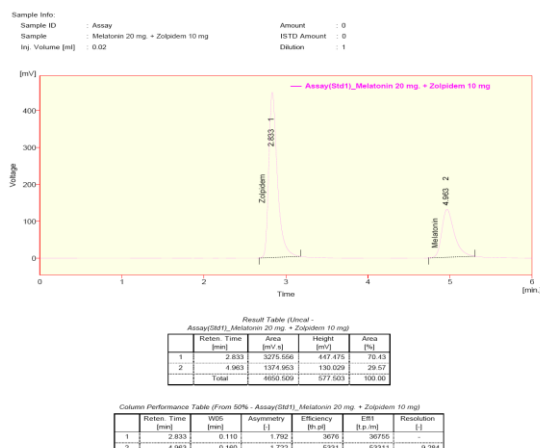


Fig: Chromatogram of Assay standard preparation-1

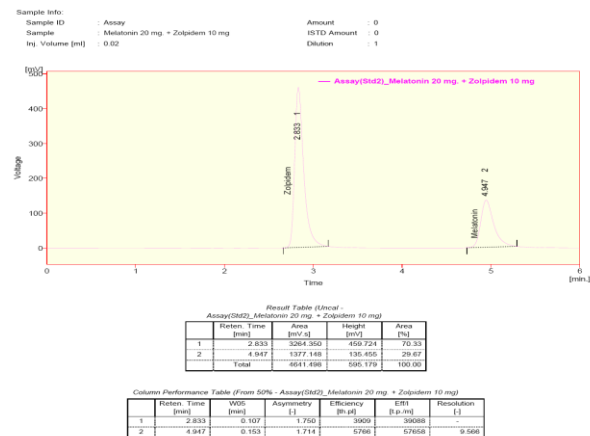


Fig: Chromatogram of Assay standard preparation-2

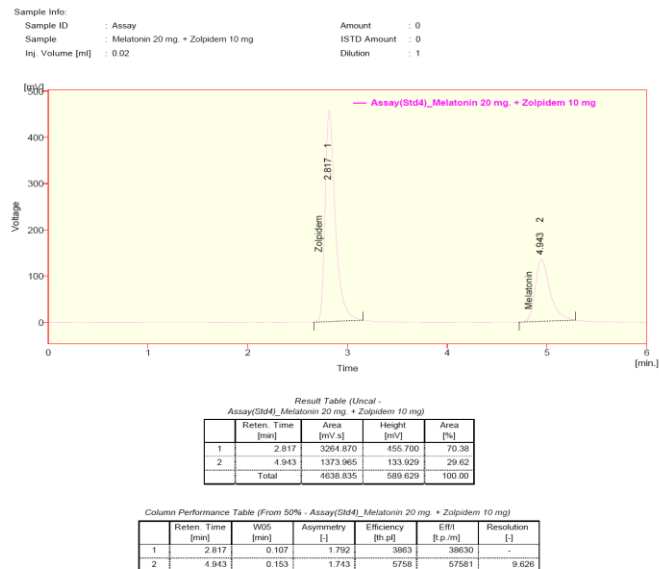


Fig: Chromatogram of Assay standard preparation-3

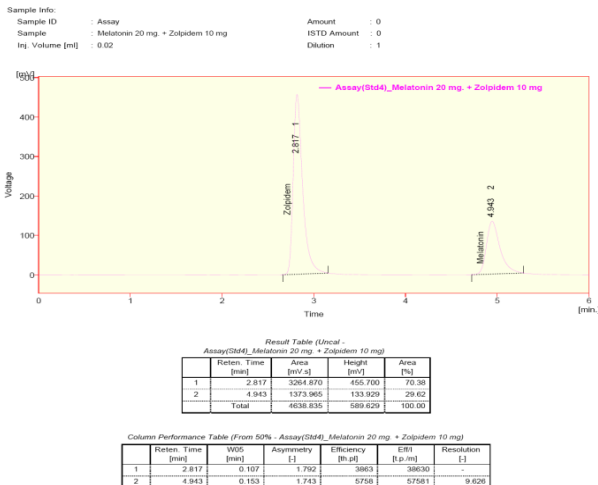
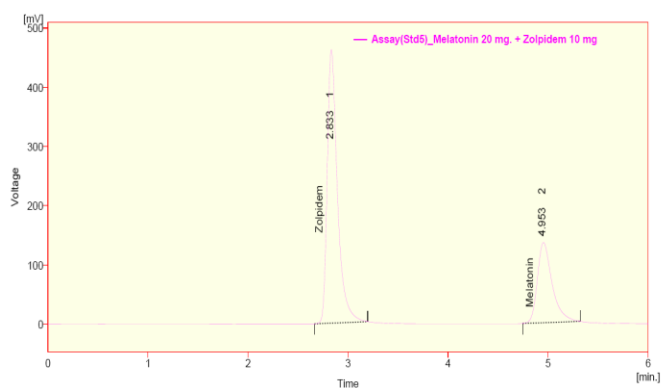


Fig: Chromatogram of Assay standard preparation-4

Sample Info:

Sample ID : Assay Amount : 0
 Sample : Melatonin 20 mg. + Zolpidem 10 mg ISTD Amount : 0
 Inj. Volume [ml] : 0.02 Dilution : 1



Result Table (Uncal -
 Assay(Std5)_Melatonin 20 mg. + Zolpidem 10 mg)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.833	3277.547	461.988	70.23
2	4.953	1389.462	135.608	29.77
Total		4667.009	597.596	100.00

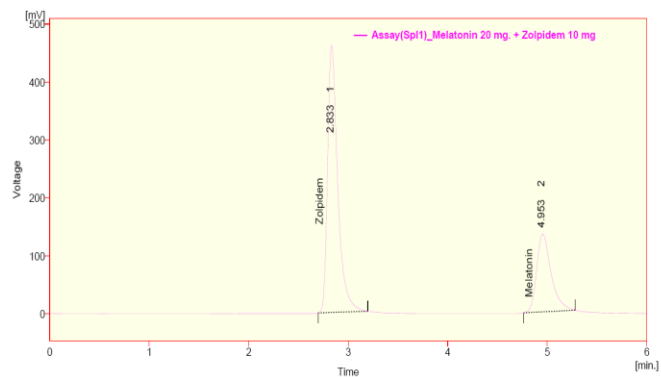
Column Performance Table (From 50% - Assay(Std5)_Melatonin 20 mg. + Zolpidem 10 mg)

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th pl]	Eff1 [t.p.m]	Resolution [-]
1	2.833	0.107	1.826	3909	39088	-
2	4.953	0.153	1.771	5781	57814	9.596

Fig: Chromatogram of Assay standard preparation-5

Sample Info:

Sample ID : Assay Amount : 0
 Sample : Melatonin 20 mg. + Zolpidem 10 mg ISTD Amount : 0
 Inj. Volume [ml] : 0.02 Dilution : 1



Result Table (Uncal -
 Assay(Spl1)_Melatonin 20 mg. + Zolpidem 10 mg)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.833	3265.567	461.418	70.64
2	4.953	1357.053	134.817	29.36
Total		4622.619	596.234	100.00

Column Performance Table (From 50% - Assay(Spl1)_Melatonin 20 mg. + Zolpidem 10 mg)

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th pl]	Eff1 [t.p.m]	Resolution [-]
1	2.833	0.107	1.826	3909	39088	-
2	4.953	0.153	1.714	5781	57814	9.596

Fig: Chromatogram of Assay sample preparation-1

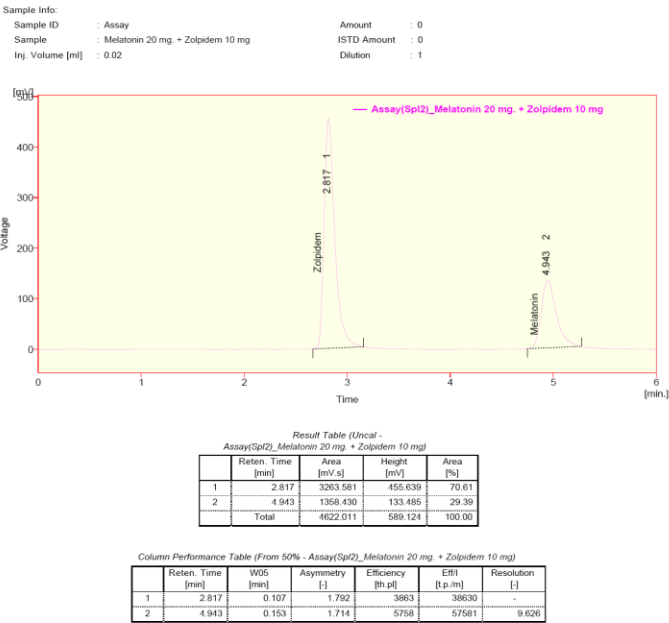


Fig: Chromatogram of Assay sample preparation-2

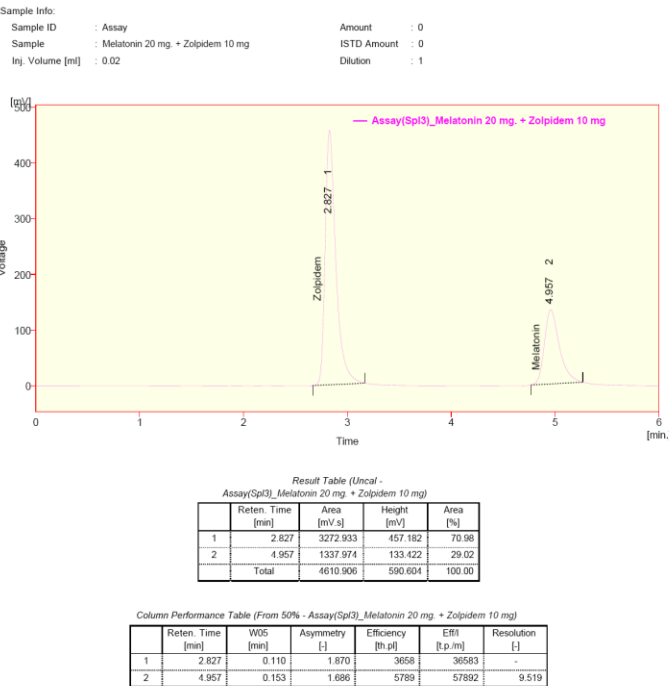


Fig: Chromatogram of Assay sample preparation-3

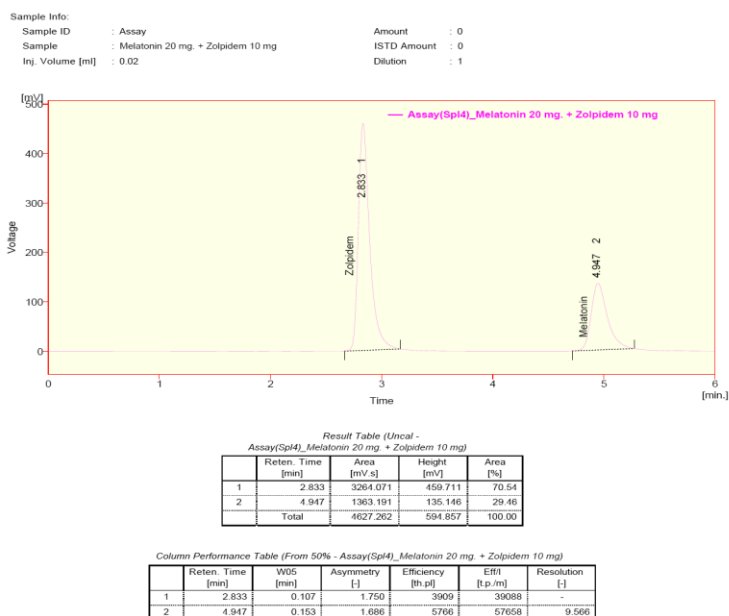


Fig: Chromatogram of Assay sample preparation-4

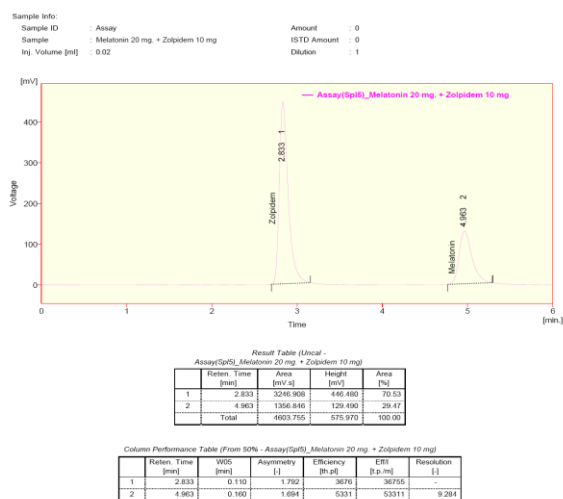


Fig: Chromatogram of Assay sample preparation-5

Table: Assay Results

MELATONIN		ZOLPIDEM TARTARATE		
	Standard Area	Sample Area	Standard Area	Sample Area
Injection-1	1374.953	1357.053	3275.556	3265.567
Injection-2	1377.148	1358.43	3264.35	3263.581
Injection-3	1367.963	1337.954	3251.196	3272.933
Injection-4	1373.965	1363.191	3264.87	3264.071
Injection-5	1389.462	1356.846	3277.547	3246.908
Average Area	1376.698	1354.695	3266.704	3262.612
Standard Deviation	9.702838		9.551679	
%RSD	0.716238		0.292762	
Assay(%purity)	98.40		99.87	

Observation

The amount of ZOLPIDEM TARTARATE and MELATONIN present in the taken dosage form was found to be **98.40%** and **99.87%** respectively.

VALIDATION

Specificity by Direct comparison method

There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

Preparation of mixed standard solution

Weigh accurately 10mg of MELATONIN and 10 mg of ZOLPIDEM TARTARATE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μ g/ml of MELATONIN

and ZOLPIDEM TARTARATE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains ZOLPIDEM TARTARATE – 10 mg, MELATONIN -20 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of ZOLPIDEM TARTARATE and MELATONIN (μ g/ml) were prepared by dissolving weight equivalent to 10 mg of ZOLPIDEM TARTARATE and 20 mg of MELATONIN and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are prepared in 5 replicates of 10 μ g/ml of ZOLPIDEM TARTARATE and MELATONIN was made by adding 1 ml of stock solution to 10 ml of mobile phase.

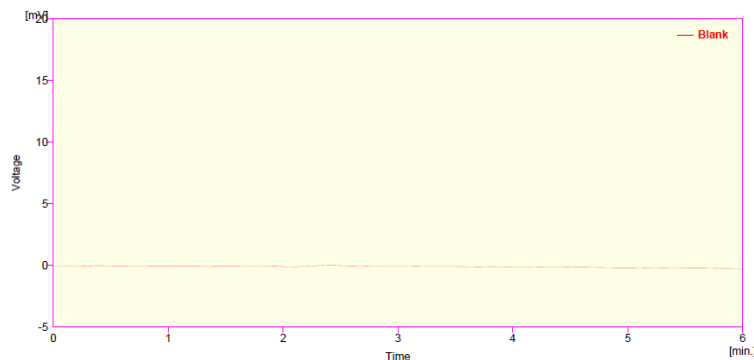


Fig: Blank chromatogram for specificity by using mobile phase

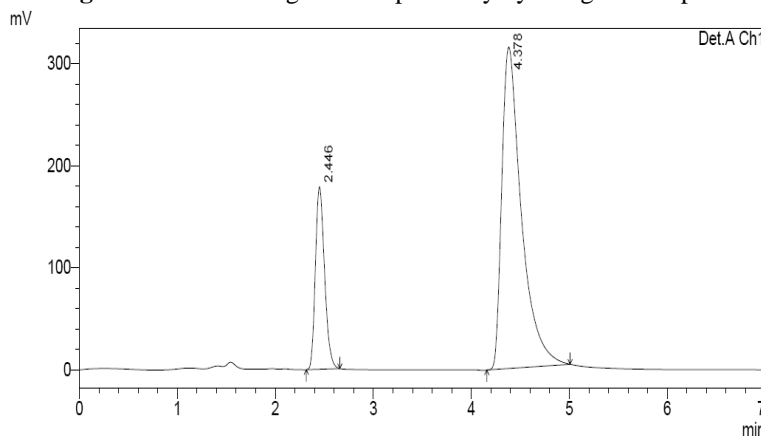


Fig: Chromatogram for specificity of ZOLPIDEM TARTARATE and MELATONIN sample

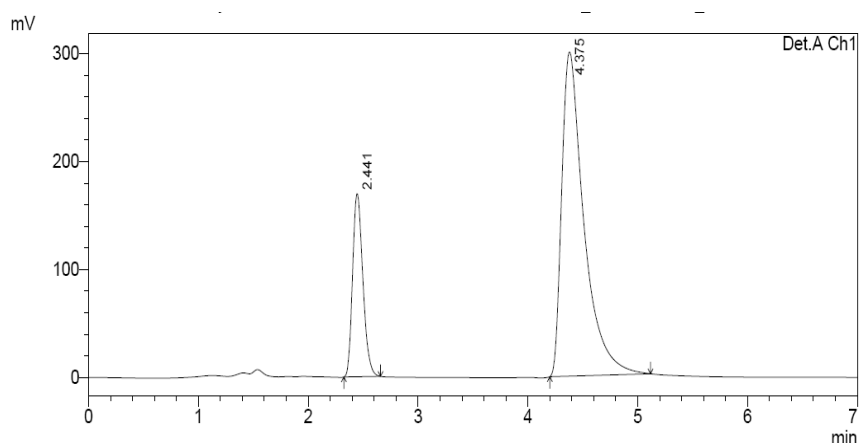


Fig: Chromatogram for Specificity of ZOLPIDEM TARTARATE and MELATONIN standard

Observation

It is observed from the above data; diluents or excipients peaks are not interfering with the ZOLPIDEM TARTARATE and MELATONIN peaks.

prepared by dissolving 60 mg of MELATONIN and 40 mg of ZOLPIDEM TARTARATE dissolved in sufficient mobile phase and dilute to 100 ml with mobile phase. Further dilutions were given in the table

Linearity and range

Preparation of standard stock solution

Standard stock solutions of MELATONIN and ZOLPIDEM TARTARATE (microgram/ml) were

Table 9.3 .1: Linearity Preparations

Preparations	Volume from standard stock transferred in ml		Volume made up in ml (with mobile phase)	Concentration of solution($\mu\text{g/ml}$)	
				MELATONIN	ZOLPIDEM TARTARATE
Preparation 1	0.2	0.2	10	20	20
Preparation 2	0.4	0.4	10	40	40
Preparation 3	0.6	0.4	10	60	60
Preparation 4	0.8	0.8	10	80	80
Preparation 5	1.0	1.0	10	100	100

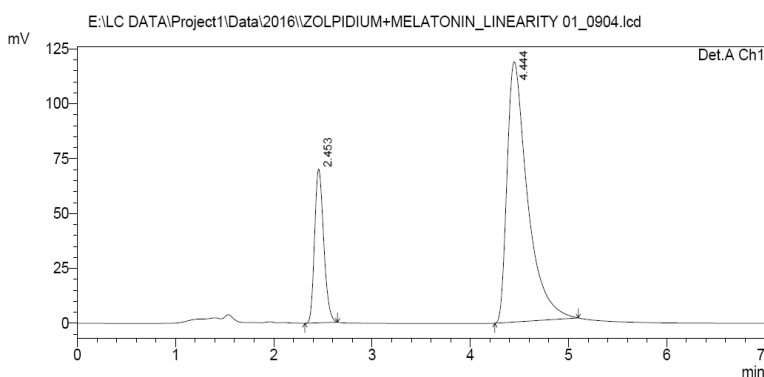


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN preparation-1

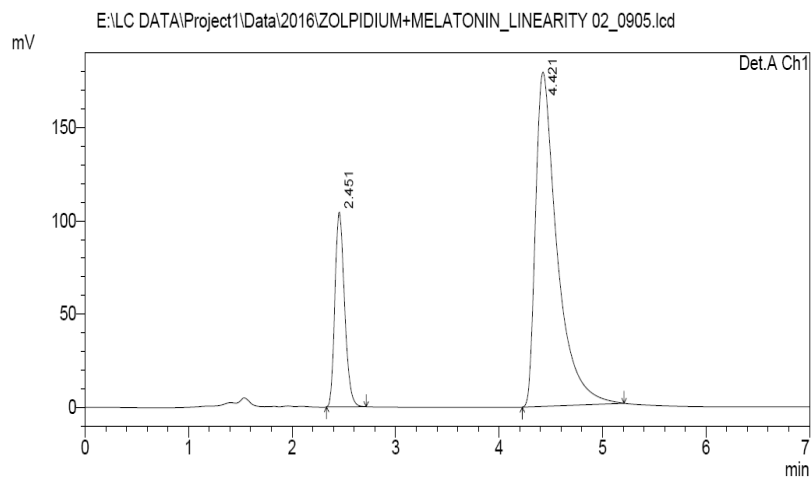


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN preparation-2

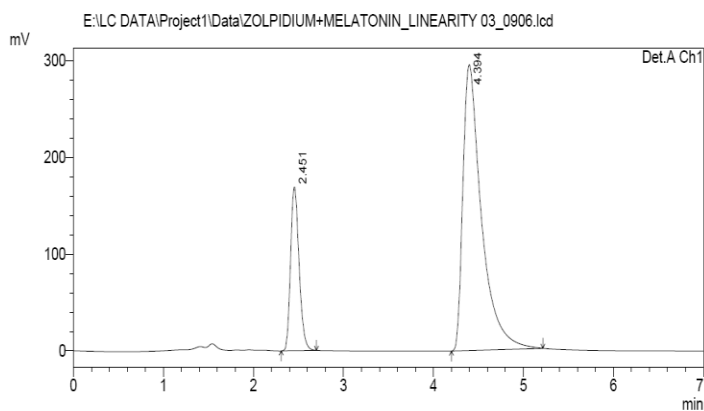


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN preparation-3

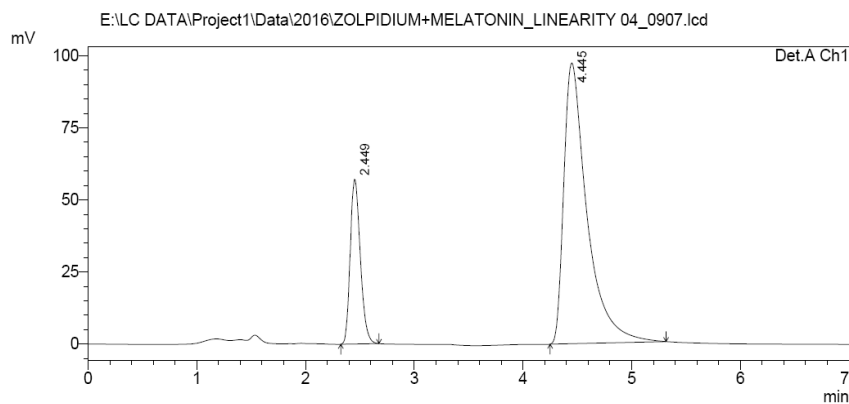


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN preparation-4

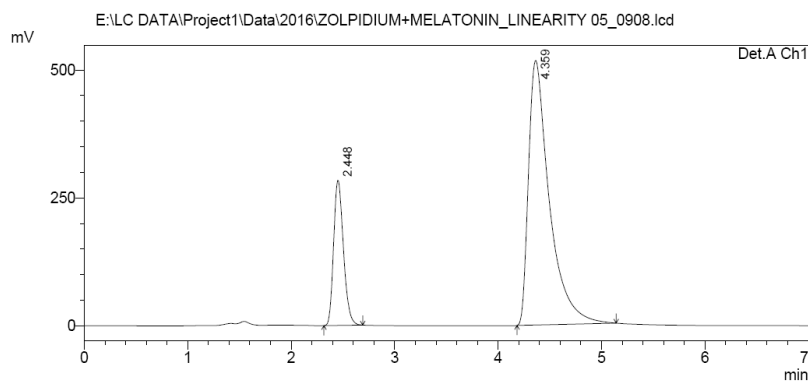


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN for preparation-5

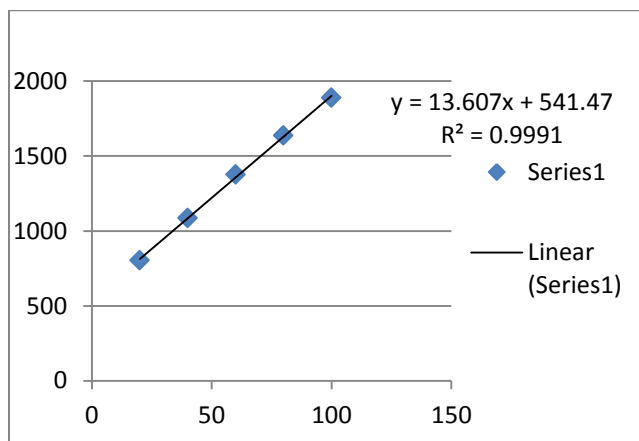
Table: linearity of MELATONIN

S. No.	Conc.(µg/ml)	Area
1	20	803.449
2	40	1085.299
3	60	1375.599
4	80	1636.606
5	100	1888.484

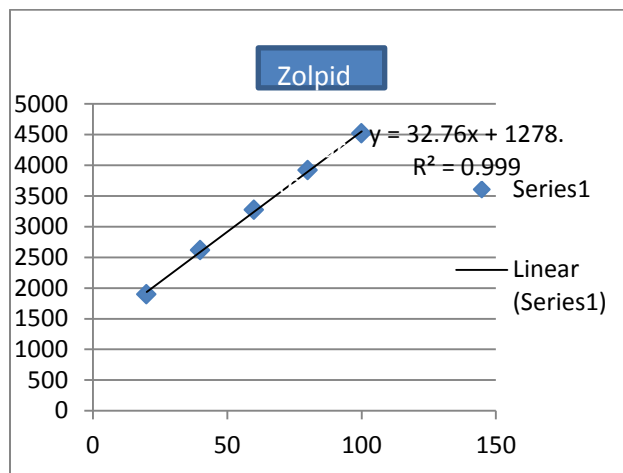
Table: linearity of ZOLPIDEM TARTARATE

S.No.	Conc.(µg/ml)	Area
1	20	1896.522
2	40	2614.218
3	60	3271.641
4	80	3919.306
5	100	4520.375

Linearity graph of MELATONIN



Linearity graph of ZOLPIDEM TARTARATE



The relationship between the concentration of MELATONIN and ZOLPIDEM TARTARATE and area of MELATONIN and ZOLPIDEM TARTARATE should be linear in the specified range and the correlation should not be less than 0.99.

Observation

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of MELATONIN and ZOLPIDEM TARTARATE is 0.996 and 0.997. The relationship between the concentration of MELATONIN and ZOLPIDEM TARTARATE and area of MELATONIN and ZOLPIDEM TARTARATE is linear in the range examined since all points lie in a

straight line and the correlation coefficient is well within limits.

Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analysed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analysed sample solution at three different levels 50%, 100%, 150%.

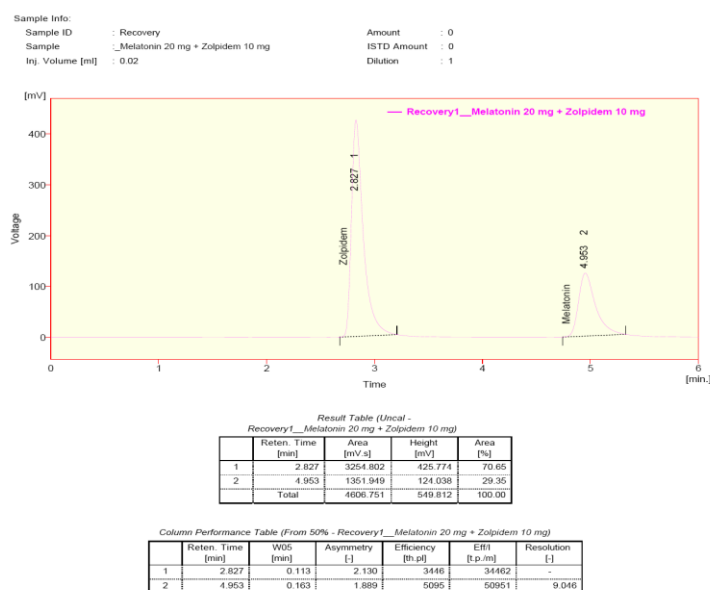


Fig: Chromatogram of 50% recovery (injection 1)

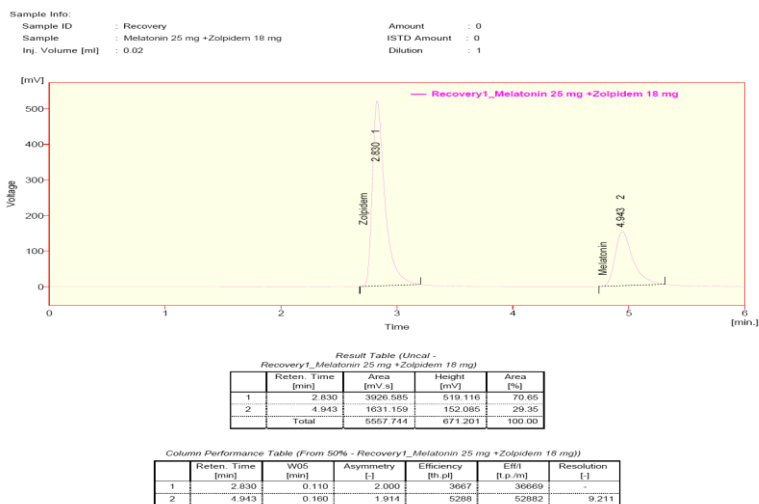


Fig: Chromatogram of 100% recovery (injection 2)

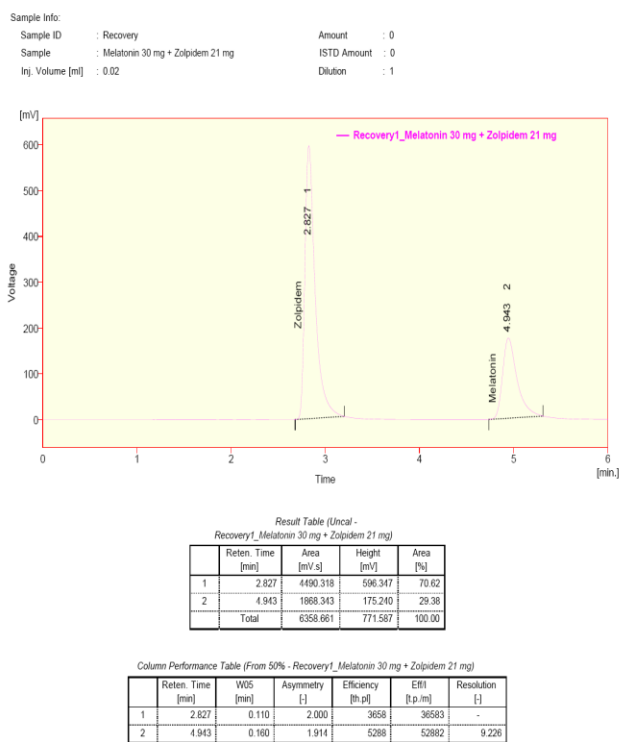


Fig: Chromatogram of 150% recovery (injection 3)

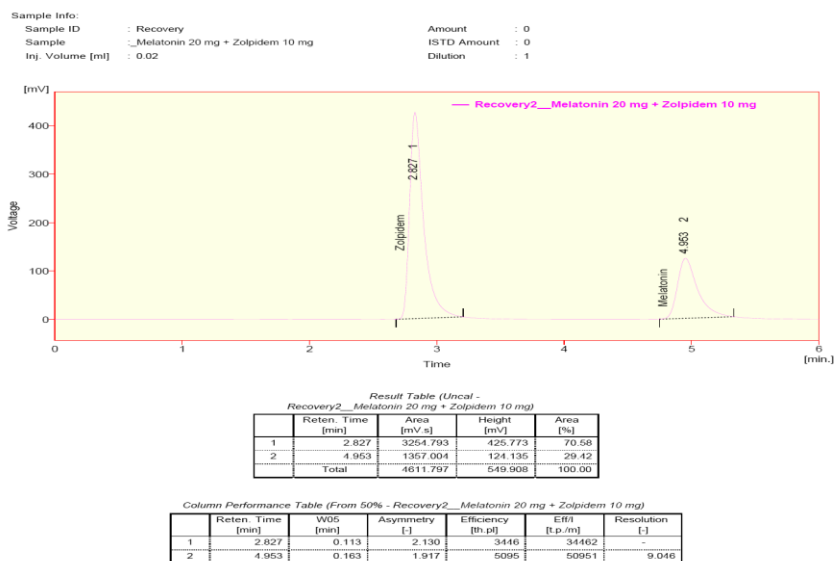


Fig: Chromatogram of 50% recovery (injection 1)

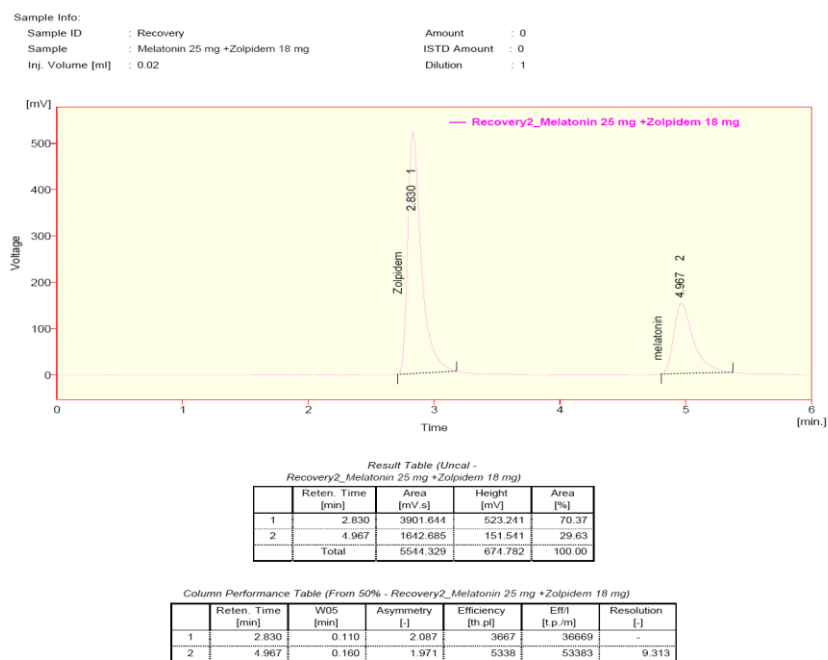


Fig: Chromatogram of 100% recovery (injection 2)

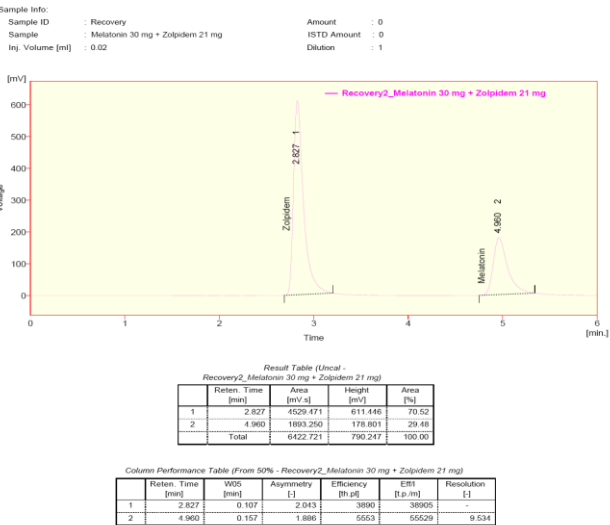


Fig: Chromatogram of 150% recovery (injection 3)

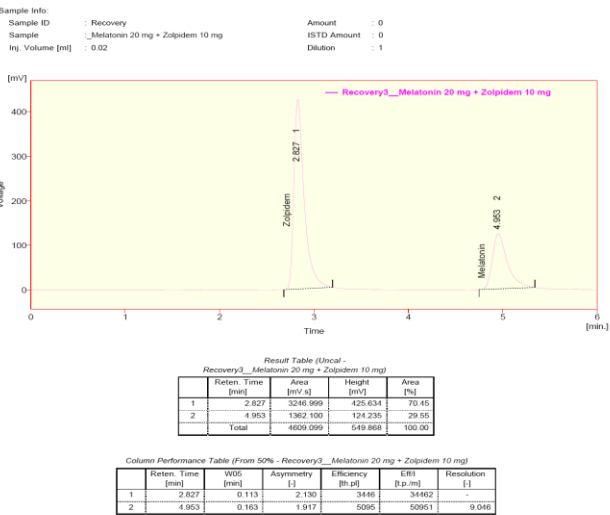


Fig: Chromatogram of 50% recovery (injection 1)

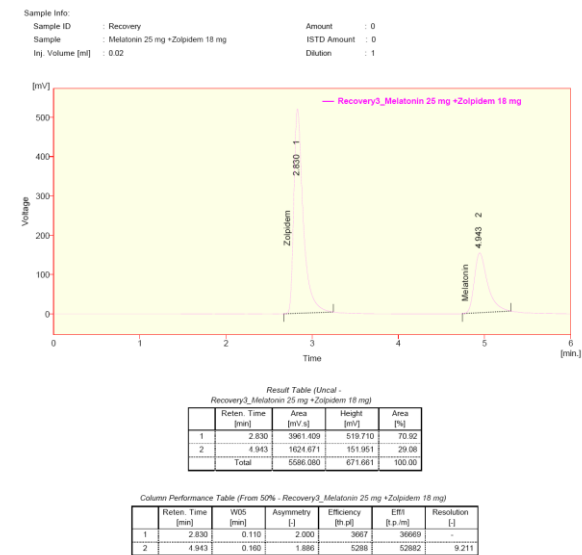


Fig: Chromatogram of 100% recovery (injection 2)

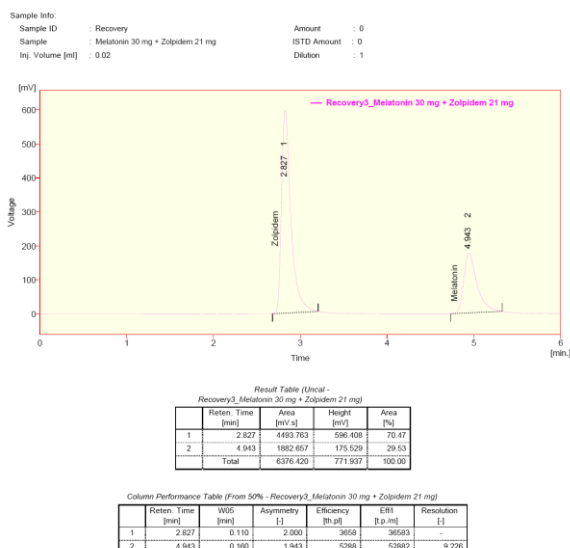


Fig: Chromatogram of 150% recovery (injection 3)

Acceptance criteria

The % recovery of ZOLPIDEM TARTARATE and MELATONIN should lie between 98% and 110%.

Table: Recovery results for ZOLPIDEM TARTARATE

Recovery level	Accuracy ZOLPIDEM TARTARATE					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
50%	20	3254.802	3252.198	19.76	99.55	100.85
	20	3254.793				
100%	20	3246.999	3929.87	25.99	120.30	
	25	3926.585				
	25	3901.644				
	25	3961.409				
150%	30	4490.318	4504.517	32.58	82.70	
	30	4529.471				
	30	4493.763				

Table: Recovery results for MELATONIN

Recovery level	Accuracy MELATONIN					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recoverd	%Recovery	
50%	20	1351.949	1357.01	19.96	99.87	110.45
	20	1357.004				
	20	1362.100				
100%	25	1631.159	1632.83	25.60	118.60	
	25	1642.685				
	25	1624.671				

150%	30	1868.343	1881.41	31.98	112.88
	30	1893.250			
	30	1882.657			

Observation

The percentage mean recovery of ZOLPIDEM TARTARATE and MELATONIN is 104.74% and 109.08 % respectively.

Acceptance criteria

The % Relative standard deviation of Assay preparations of ZOLPIDEM TARTARATE and MELATONIN should be not more than 2.0%.

PRECISION

Method precision

Prepared sample preparations of ZOLPIDEM TARTARATE and MELATONIN as per test method and injected 6 times in to the column.

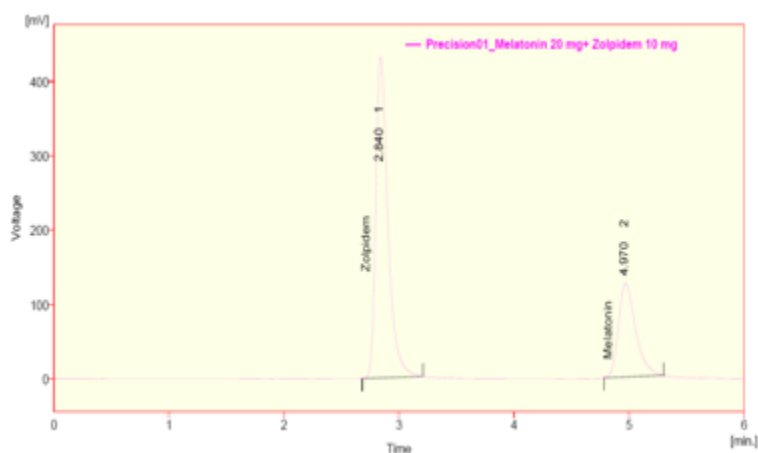


Fig: Chromatogram of precision injection 1

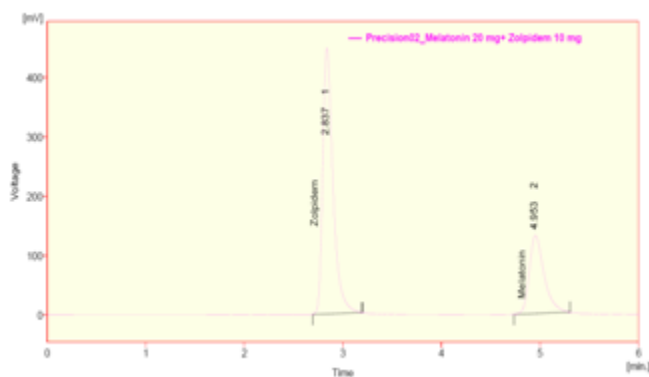


Fig: Chromatogram of precision injection 2

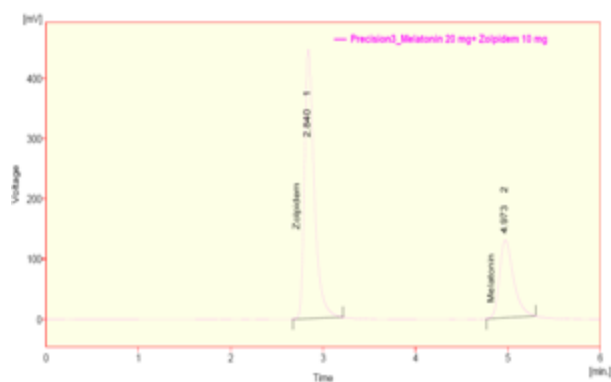


Fig: Chromatogram of precision injection 3

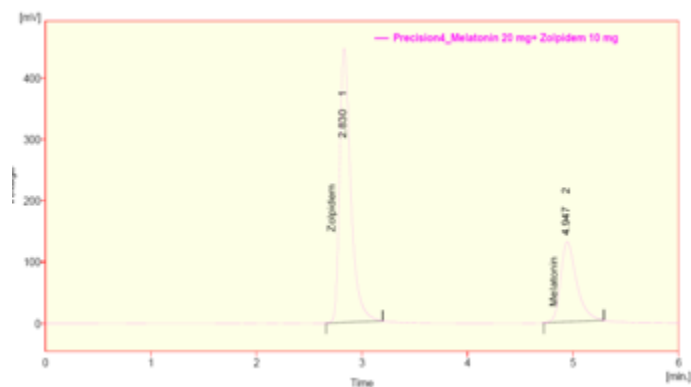


Fig: Chromatogram of precision injection 4

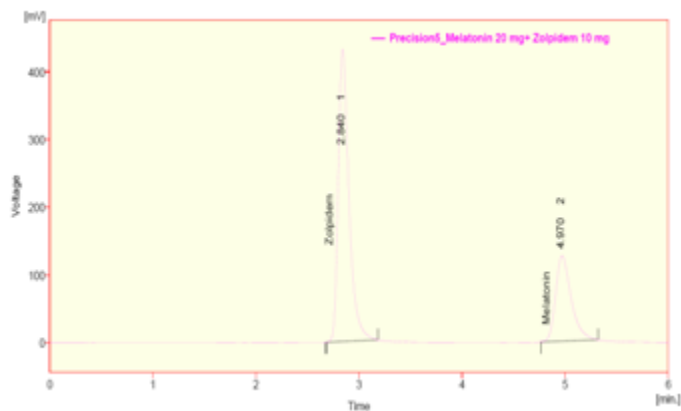


Fig: Chromatogram of precision injection 5

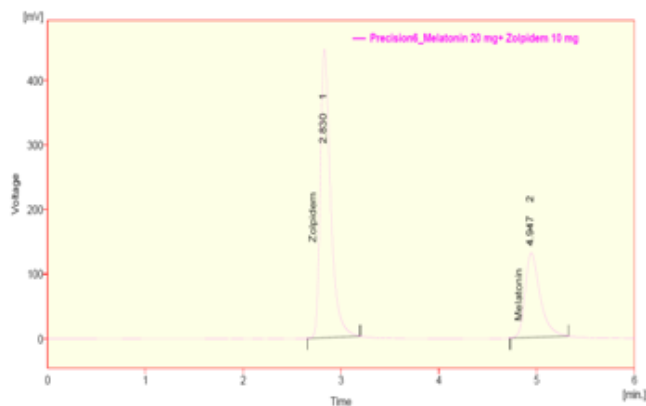


Fig: Chromatogram of precision injection 6

Table: Results for precision of ZOLPIDEM TARTARATE and MELATONIN

MELATONIN			ZOLPIDEM TARTARATE		
S.No.	Rt	Area	S.No.	Rt	Area
1	4.97	1305.008	1	2.84	3146.809
2	4.953	1378.279	2	2.837	3262.452
3	4.973	1360.268	3	2.84	3294.392
4	4.947	1377.65	4	2.83	3283.112
5	4.97	1325.322	5	2.84	3132.277
6	4.947	1397.749	6	2.83	3283.16
avg	4.96	1357.379333	avg	2.836167	3233.7
stdev	0.012296341	-	stdev	0.004916	-
%RSD	0.247910099	-	%RSD	0.173331	-

Observation

Test results for MELATONIN and ZOLPIDEM TARTARATE are showing that the %RSD of Assay results are within limits.

different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision.

Acceptance criteria

The system suitability should pass as per the test method at variable conditions.

ROBUSTNESS

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared solution as per test method and injected at

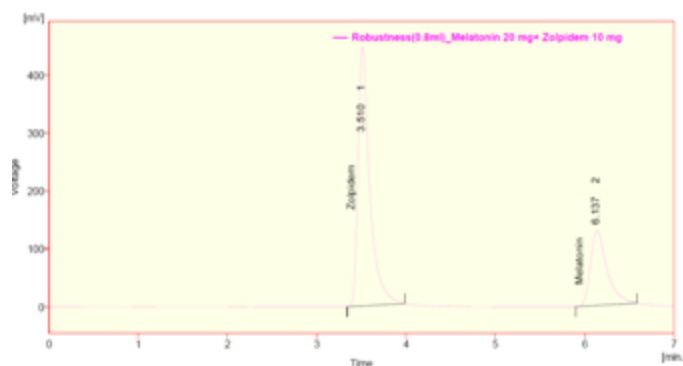


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN Robustness (0.8 ml/min)

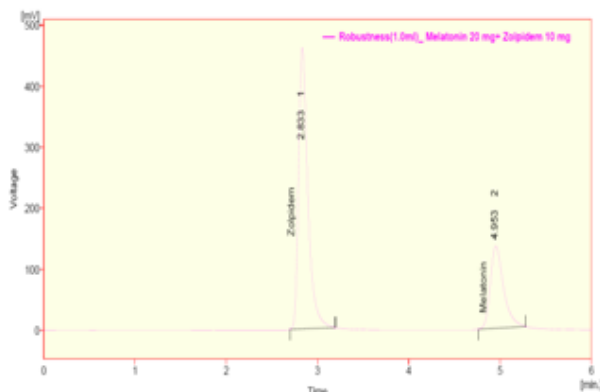


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN for Robustness (1.2 ml/min)

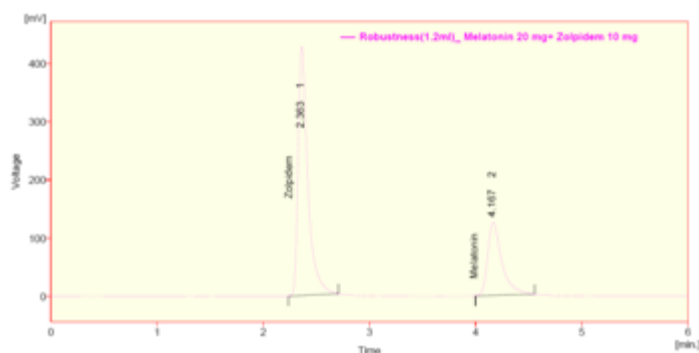


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN for Robustness (278)

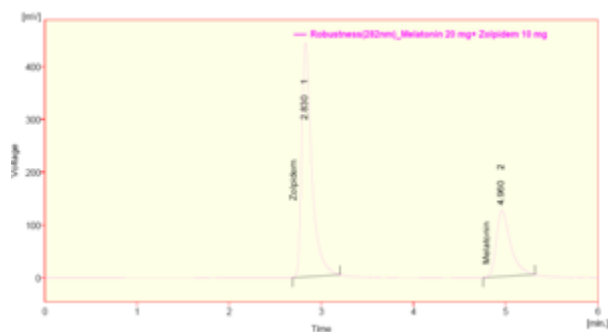


Fig: Chromatogram of ZOLPIDEM TARTARATE and MELATONIN for Robustness (282)

Observation

From the observation it was found that the system suitability parameters were within limit at all variable conditions.

Ruggedness

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts

Acceptance criteria

The % Relative standard deviation of Assay values between two analysts should be not more than 2.0%.

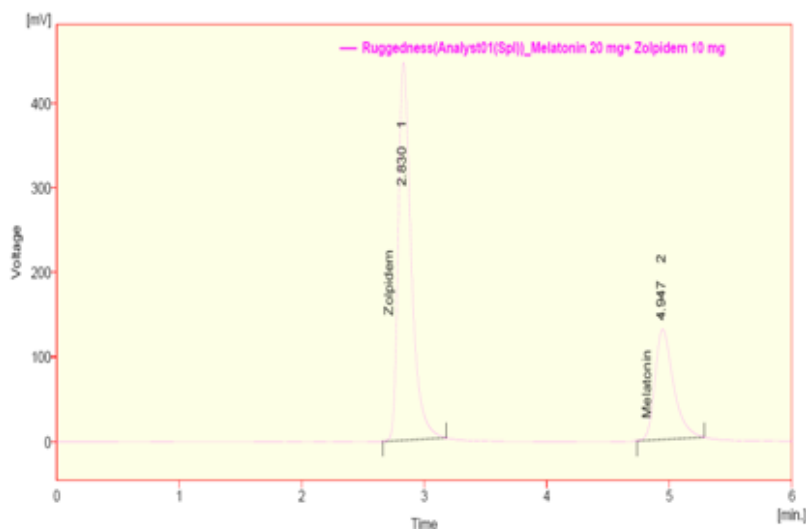


Fig: Chromatogram of Analyst 01 standard preparation

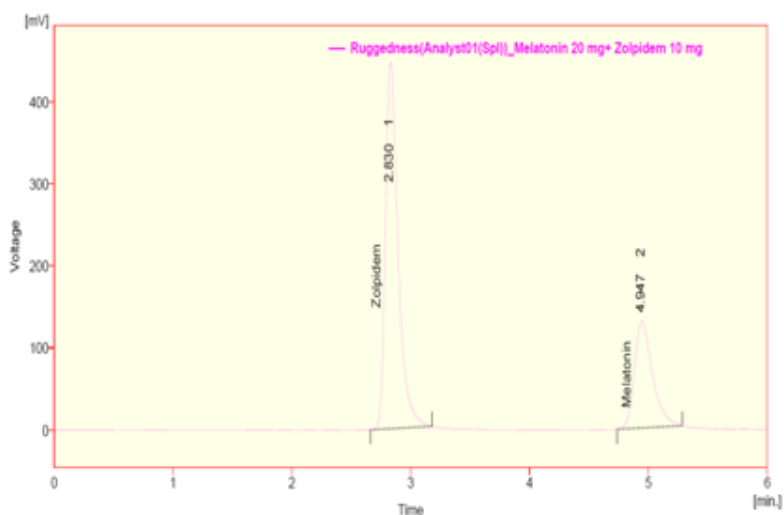


Fig: Chromatogram of Analyst 01 sample preparation

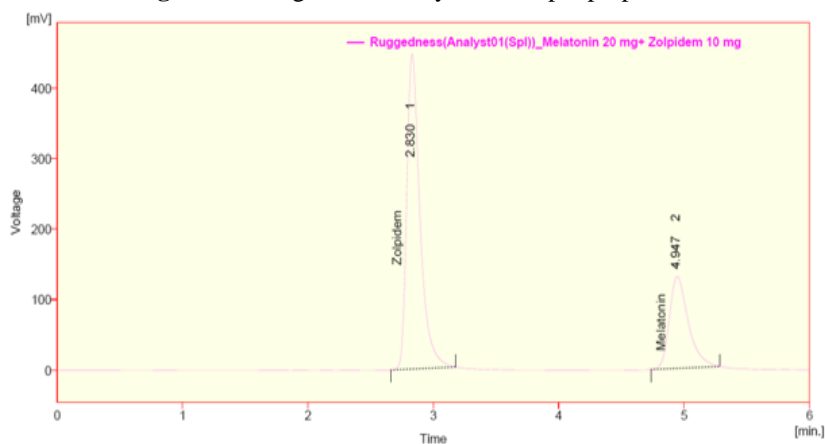


Fig: Chromatogram of Analyst 02 standard preparation

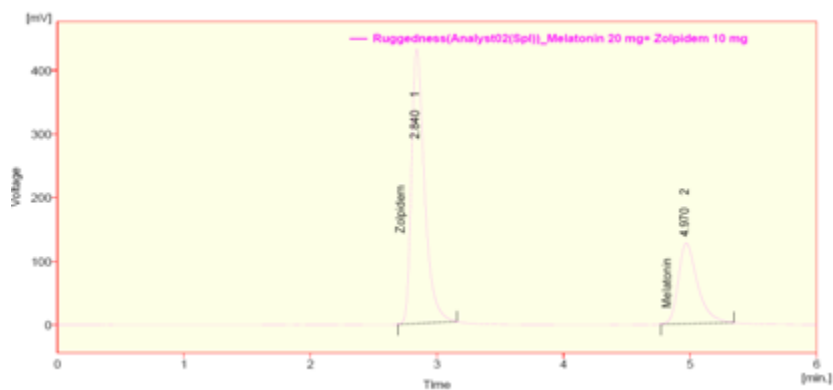


Fig: Chromatogram of Analyst 02 sample preparation

Table 9.9.5: Results for Ruggedness

MELATONIN	%Assay	ZOLPIDEM TARTARATE	%Assay
Analyst 01	100%	Analyst 01	99.37%

From the observation the %RSD between two analysts Assay values not greater than 2.0%, hence the method was rugged.

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