"Method Development and Validation for Estimation of Melatonin in Oro- Dispersible Effervescent Tablet By RP-HPLC"

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ABSTRACT

In the present research work, successful attempt was made for Estimation MEL in Orodispersible Effervescent tablet using HPLC method. The simplicity, rapidity, reproducibility and economy of proposed method completely fulfil the objective of this research work.HPLC method was developed and validated as per ICH guideline. Developed method was found to be simple, precise and accurate. Using Column C18 Hypersil ODS. The mobile phase was composed of methanol: phosphate Buffer (40:60), detection was done at 223nm. Retention time was found to be 7.1min.There were no methods for estimation of Melatonin in Oro dispersible effervescent tablet. The following method had been developed for estimation of Melatonin in effervescent formulation.Method Development and Validation for Estimation of Melatonin in Effervescent Tablet by RP-HPLC.A new RP-HPLC method was developed for assay of Melatonin in formulation. The separation was achieved on column C18 ODS (Hypersil), 250 nm×4.6 mm, 5µm using methanol: Phosphate Buffer (40:60) use as mobile phase for estimation of Melatonin. Detection was carried out in UV detector at 223 nm.The retention time and total run time was found to be stable at 7.1 min (RT), flow rate1.0 ml, Oven temperature is 25 °C and Injection volume was 20 µl.The developed RP-HPLC method was validated for linearity, accuracy, precision, intraday precision, limit of detection and limit of quantitation as per ICH guideline. Developed method was accurate, precise robust and rugged, it showed % RSD not more than 2%.

Keywords: *Melatonin, Oro- Dispersible, Effervescent Tablet, RP-HPLC*

INTRODUCTION

1.1 Analytical Method

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. Analytical Chemistry is well-defined as "The science and the art of determining the composition of materials in terms of the elements or compounds contained.**[1]**

- Analytical methods which are amount of quality of the drugs play a very comprehensive role in drug development and follow up activities. It assures that a drug product meets the established standard, is stable and will continue to meet purported quality throughoutits shelf life.**[2]**
- Pharmaceutical analysis plays an important role right from the testing of raw materials; in-process quality checks and analysis of finished products. Pharmaceutical analysis is considered to determine the identity, strength, quality and purity of drug samples. **[3]**
- In analytical chemistry, it is of prime importance to gain information about the qualitative and quantitative compositions of substances and chemical species, that is, to find out what a substance is composed of and exactly how much. In general terms, pharmaceutical analysis comprises of those procedures necessary to determine the "identity, strength, quality and purity" of drugs. Pharmaceutical analysis is a branch of chemistry involving a process of identification, determination, quantification, purification and separation of

components in a mixture or determination of chemical structure of compounds.[4]
There are two main types of analysis – Qualitative and Quantitative analysis.

1. Qualitative analysis is performed to establish composition of a substance. It is done todetermine the presence of a compound or substance in a given sample or not. The various qualitative tests are detection of evolved gas, limit tests, colour changereactions, determination of melting point and boiling point, mass spectroscopy, determination of nuclear half-life etc.[5]

Materials and Methods :

List of Equipment / Instruments:

The following equipment and instruments have been used in the present studies.

Table List of Instruments

Name of Instrument	Manufacture
UV-Visible Spectrophotometer	Shimadzu UV 1800 240V
HPLC	Agilent 1260 LC infinity
Ultra Sonicator	Vila Sonicator
pH meter	Toshcon
Analytical balance	Aczet
HPLC Column	Hypersil (250 mm× 4.6 mm)
HPLC Software	EZ chrome lite

6.1 Chemicals and Solvents

The following Chemical and Solvents have been used in the present studies

Table No..: Chemicals and Solvents

Sr.No.	Chemical	Manufactured by
1	HPLC Grade Water	Siddhi Lab
2	Methanol	Rankem
3	Ortho Phosphoric Acid	Rankem
4	Potassium dihydrogen Phosphate	Rankem

EXPERIMENTAL WORK:

Preparation of Solution:

Preparation of Diluent: 100% Mobile phase

Preparation of Melatonin Standard: Accurately 10 mg Melatonin was weighed anddissolved in 100

ml mobile phase, shook well to mix and sonicated for 5 minutes, filtered through 0.2-micron filter Paper and used filtrate for injection.

Preparation of Sample solution: Equivalent to 10 mg Melatonin was weighed form crushed Melatonin tablets powder. Added 30 ml of the mobile phase for 5 minutes, added sufficient of the mobile phase to produce 100 ml. Shook well to mix and sonicated for 5 minutes, filtered through 0.2-micron filter Paper and use filtrate for injection.

Identification of drug: -

- **Melting point determination-** Identification of melting point of MEL was done by using digital melting point determination instrument. Result is mentioned in **Table**
- **Solubility analysis-** Solubility of MEL was checked by dissolving it in numberof solvents, like HPLC water, Methanol, Acetonitrile and Buffer. Result is mentioned in
- **Fourier transform Infra-red Spectroscopy (FTIR):** Infrared absorption spectrum of Melatonin was recorded over the wave number 2000 to 400 cm^{-1} using FourierTransform spectrophotometer (Agilent Technologies). FTIR Spectra is represented in **F**.

Mobile Phase Preparation:

- **Preparation of phosphate buffer pH 3.0:**

Accurately 6.8 gm of Potassium dihydrogen phosphate was weighed and dissolved in 1000 mL of water. The pH was adjusted 3.0 0.02 using Ortho phosphoric acid. The buffer was filtered through 0.22µm filters to remove all fine particles and gases.

Preparation of mobile phase:

The mobile phase was prepared by mixing the buffer and methanol in the ratio of 60:40, sonicated for 5 min. After the solution was filtered through 0.22 µm to remove all fine particles and gases.

METHOD DEVELOPMENT FOR ASSAY

1. Trial 1:

In this trial ACN was selected as a mobile phase and standard Melatonin solution of 10 ppm was prepared in the selected mixture, then chromatogram was recorded afterinjecting the sample in HPLC system. Chromatographic condition mention in **Table**

Table : Chromatographic Condition for Trial 1:

Column	Hypersil C18
Elution mode	Isocratic
Mobile phase	ACN
Flow rate	1.5 mL /min
Detection wavelength	223 nm
Injection volume	10 µL
Run time	10 min
Column temp	25 ^{0}C

7.6 Analytical Method Validation:

1. System suitability: System suitability test are used to verify that the given method ofanalysis is adequate for the intended analysis.

Procedure: Standard preparation: Make the 10 µg/mL of MEL Stock Solution usingmobile phase.

Acceptance criteria

1. The % RSD for the retention times of melatonin Peaks from 5 replicate injections ofeach Standard solution should be not more than 2.0
2. The % RSD for the peak area responses of melatonin peaks from 5 replicate injectionsof each standard solution should be not more than 2.0%.
3. The number of theoretical plates (N) for the melatonin peaks is not less than 2000.

4. The Tailing factor (TP) for the Melatonin peak is not more than 2.0.Result of System suitability is mentioned in **Table No.8.4**

2. Linearity: The linearity of analytical procedure is its ability to obtain test results whichare directly proportional to the concentration of analyte in sample

- Preparation of standard stock solution

Standard stock solutions of Melatonin (100µg/mL) were prepared by dissolving 10 mg ofmelatonin in 100 mL of mobile phase. Sonicated for 2 min. further dilutions were given in **Table No.7.5** and Result is mentioned in **Table N**

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3.RESULT AND DISCUSSION:

Identification of drug: -

Melting point determination:

Table No. 8.1: Result of Melting Point

Sr.No.	Melting Point	
	Literatrue	**Observed**
1	116-118^{0}c	117.6^{0}c

Discussion- Melting Point of melatonin was found to 117.6^{0}c. That show its Purity.

Solubility Analysis:

Table No. 8.2: Result of Solubility

Sr.No.	Ingredients	Solubility
1	HPLC Water	Slightly soluble in water
2	Methanol	Freely Soluble in Methanol
3	Acetonitrile	Freely solublein Acetonitrile
4	Buffer (Phosphate buffer pH 3)	Freely solublein Buffer.

Discussion- From above observation methanol, acetonitrile and buffer showed goodsolubility for melatonin hence Methanol, Acetonitrile and buffer selected as commonsolvent for proper elution in RP-HPLC.

Fourier transform Infra-red Spectroscopy (FTIR):

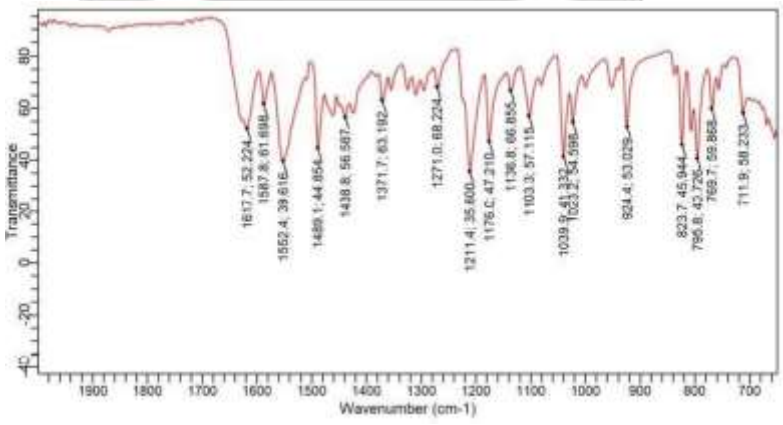


Fig. 8.1: FTIR Spectra of Melatonin Table No.

Sr.No.	Functional Group	Standard Range (cm-1)	Observed Value (cm-1)
1	N–H bend	1650-1580	1617
2	C=C Stretch	1450-1200	1438
3	C=O stretch	1300-1000	1271
4	C-O stretch	1250-1050	1211,1176
5	C-H bend	860-680	769

8.3: Result of FTIR Spectra

Discussion- The Fourier transform infrared spectroscopy (FTIR) spectrum for the pureMelatonin Drug show all Functional group was Present in **Table No.8.3**

8.1 Working Wavelength (λmax):

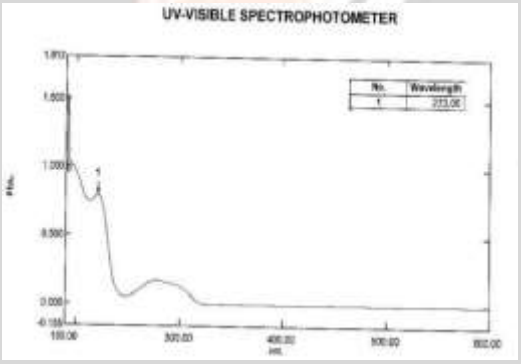


Fig.8.2: UV Spectra of melatonin Discussion:

Working Wavelength of melatonin was reported 223nm.

8.2 Method Development for Assay Trial:

Trial 1:

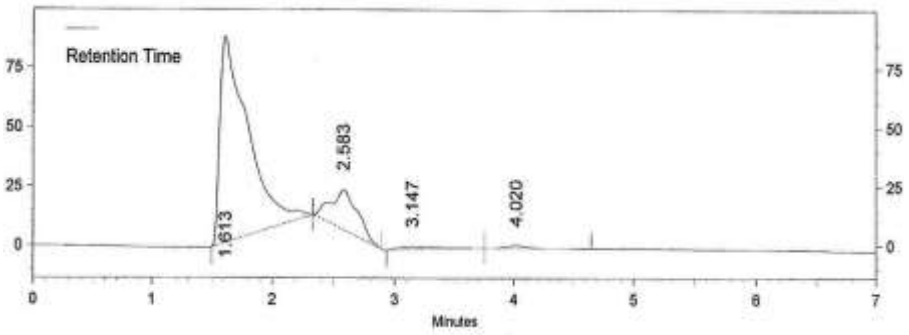


Fig.8.3: Chromatogram Obtained from Trial 1

Observation: Improper peak shape

Discussion: Due to specificity reason above method cannot be used, further trial to becarried auto proper separation by changing mobile phase composition.

8.3.1 Trial 2 :

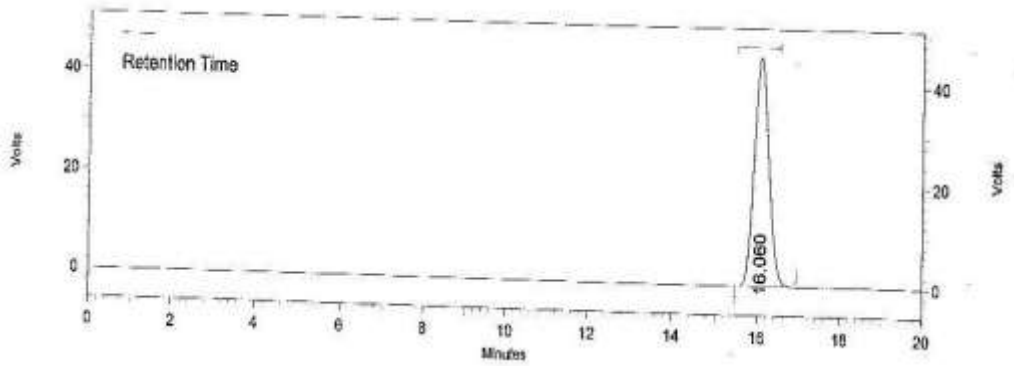


Fig.8.4: Chromatogram Obtained from Trial 2

Observation: Peak observed at retention time 16.060 minute.

Discussion: Due to longer retention time above method cannot be used. Further trialsneed to be carried out to shorten retention time.

Trial 3 :

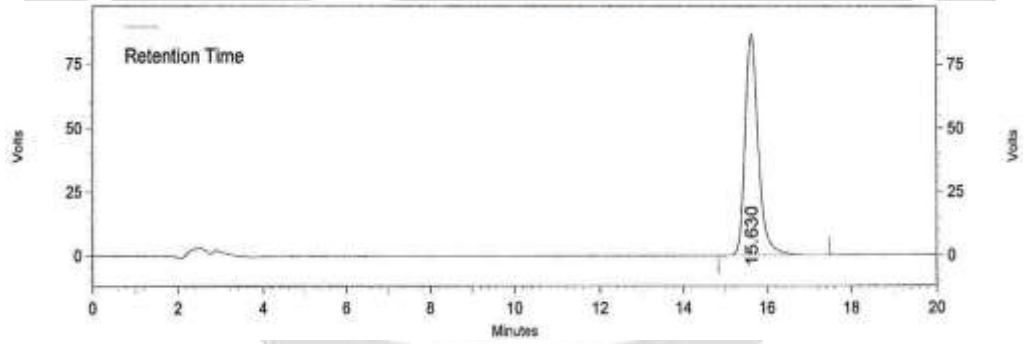


Fig.8.5: Chromatogram Obtained from Trial 3

Observation: Peak observed at retention time 15.630 minute.

Discussion: Due to longer retention time above method cannot be used. Further trialsneed to be carried out to shorten retention t

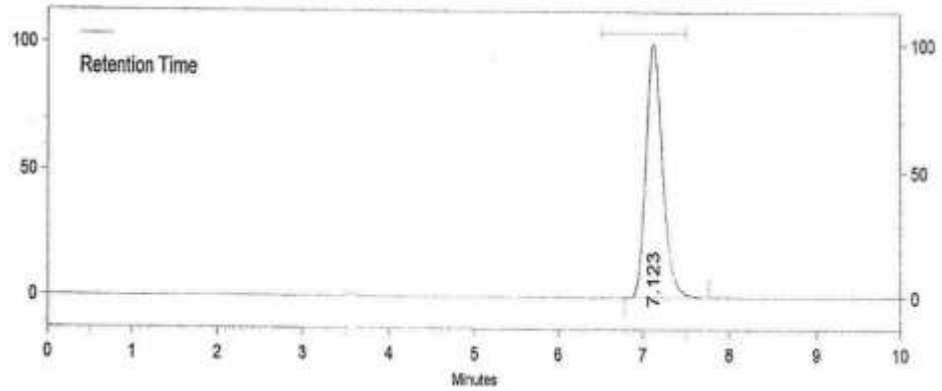


Fig.8.6: Chromatogram Obtained from Trial 4

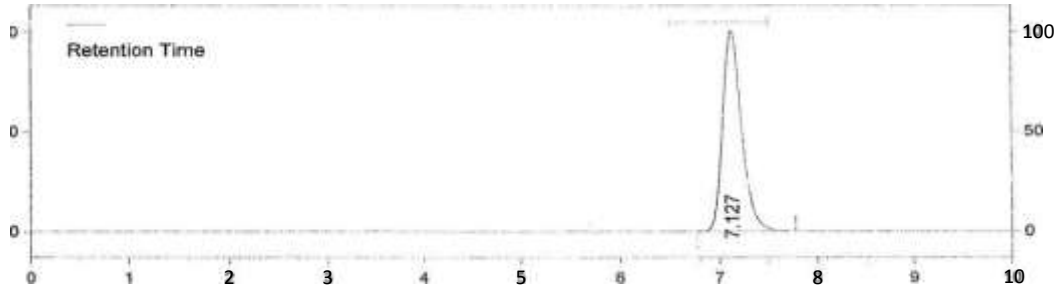
Observation: Above peak was found to be satisfactory.

Discussion: So, condition of fourth trial were selected as optimised chromatographiccondition. It reduces the cost.

System Suitability: System suitability test are used to verify that the given methodof analysis is adequate for the intended analysis. Results are summarized in **Table No.8.4**and chromatogram Represented in **Fig 8.7.**

Table No.8.4: Observation for system suitability

Sample No.	**Peak area**	**Asymmetry Factor**	**Theoretical Plate**	**Retention Time**
Sample 1	22760802	1.25529	6525	7.130
Sample 2	22766171	1.26024	6507	7.127
Sample 3	22760873	1.27385	6493	7.123
Sample 4	22757274	1.26555	6530	7.123
Sample 5	22763060	1.25183	6562	7.123
Mean	22761636	1.261352	6523	7.1252
Std	3274.86	----	----	0.0031
% RSD	0.0143	NMT 1.5	NLT 2000	0.044



Chromatogram Obtained from system suitability Conclusion: The plate count and tailing factor results were found to be satisfactory and are found to be within the limit. The % RSD was found to be 0.0143.

Discussion: System suitability solution was found within the limit so that the givenprocedure of analysis is suitable on above mentioned chromatographic conditions.

Linearity:

Table No.8.5: Observation for Linearity

Conc. (PPM)	AREA
2	5241704
4	10288305
6	15460269
8	20508193
10	26713649
12	30504235
SLOPE	2580523.014
Y- INTERCEPT	55731.4
COREELATION	0.9988

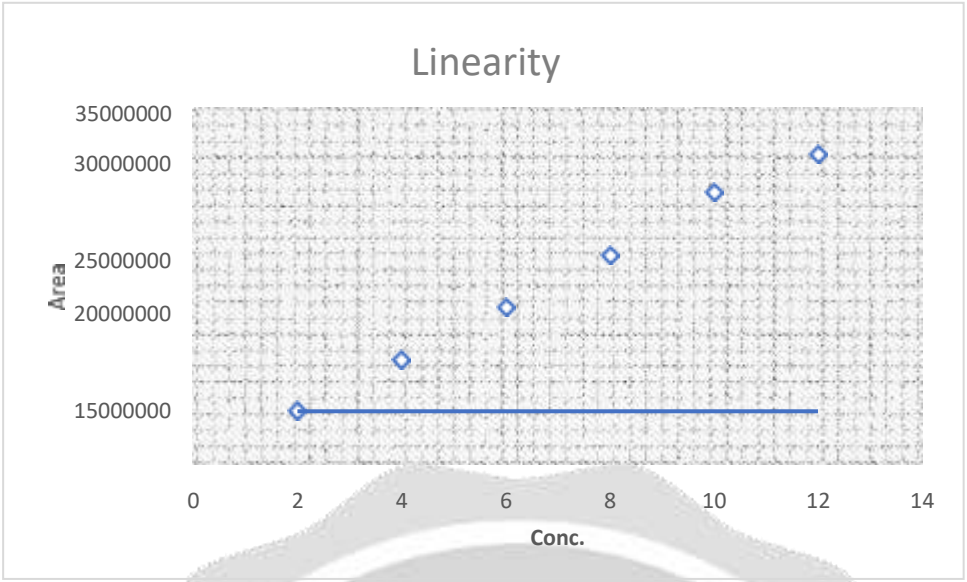


Fig.8.8: Graph of linearity

Acceptance criteria:

The correlation coefficient is not less than 0.99.

Conclusion:

The correlation coefficient for linear curve obtained between concentration vs. Area forstandard preparations of melatonin was found to be 0.998 respectively.

Discussion: The observed values were within the acceptance criteria. The Graph oflinearity show the linear.

8.4.3. Precision:

A. Repeatability:

Table No. 8.6: Observation for Repeatability

Sample	**% Assay**
Sample 1	99.22
Sample 2	98.73
Sample 3	98.81
Sample 4	98.62
Sample 5	98.79
Sample 6	98.61
Mean	98.80
Std Dev.	0.2235
%RSD	0.2263

Conclusion:

1. Assay of melatonin sample was found in the limit.
2. As per acceptance criteria RSD of % assay of six samples is NMT 2.0 % it comes0.22% .

B. Intermediate precision:

Table No. 8.7: Observation for Intermediate Precision

Days	Sample	% Assay of Melatonin
Day 1	Sample-1	98.39
	Sample-2	99.05
	Sample-3	98.55
	Average	98.66
	Std Dev.	0.344287
	% RSD	0.3489
Day 2	Sample-1	100.25
	Sample-2	99.69
	Sample-3	98.77
	Average	99.57
	Std Dev.	0.747262
	% RSD	0.7504

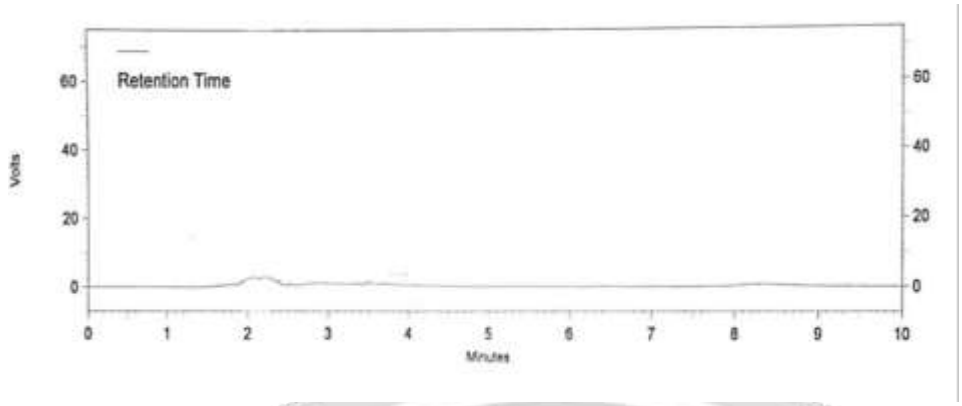
Conclusion:

1. Assay of Melatonin sample was found in the limit.
2. % RSD of Assay was within limit i.e., NMT 2.0%

Discussion- From the above data and assays are within specified limits, which indicates that the procedure for analysis was precises for the given set of chromatographic conditions.

Assay Spl 1	80.50	98.36	122.56
Assay Spl 2	80.63	98.28	122.05
Assay Spl 3	81.09	98.49	121.10

Mean % Assay	80.74	98.37	121.90
% Recovery Spl 1	100.62	98.36	102.1
% Recovery Spl 1	100.78	98.28	101.70
% Recovery Spl 1	101.36	98.49	100.91
Mean % Recovery	100.92	98.37	101.58
SD	0.3179	0.08653	0.4944
%RSD	0.315	0.08797	0.4867



Observation for Placebo

Limit of detection (LOD) and Limit of Quantification (LOQ):

Observation for LOD and LOQ

Parameters	**Result**
SD of intercept	15133.77
Slope	2580523.01
LOD (µg/mL)	0.019µg/mL
LOQ (µg/mL)	0.058µg/mL

Discussion: From the above observation LOD and LOQ was found 0.019µg/mL and0.058µg/mL.

Specificity and Placebo study:

- **Placebo sample:**

Sample of placebo was weighted and transferred into volumetric flask. Volume was madewith mobile phase.

Table No.8.10: Observation for Placebo

Sample No.	**Wt. taken. (Placebo) gm**	**Peak area**	**% Assay**
Placebo 1	0.135	N.D.	-----
Placebo 2	0.135	N.D.	-----
Placebo 3	0.135	N.D.	-----

	Mean	----- -	-----
	Std Dev.	----- -	-----
	% RSD	----- -	-----

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