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Analytical Method Development and Validation of Melatonin by QbD Approach Form

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ABSTRACT

The scientific way to develop a simple and robust analytical HPLC method for the critical separation is QbD approach. Quality-by-design (QbD) is a systematic approach to product or process development, which begins with predefined objectives, and uses science and risk management approaches to gain product and process understanding and ultimately process control. The concept of QbD can be extended to analytical methods. A simple analytical method was developed and used to identify and quantify the active pharmaceutical ingredients melatonin. In the present work, two independent factors were used such as flow rate (A), wavelength (B). Totally 17 experimental runs were suggested by the software for analyzing the interaction of each level on formulation characters and the peak area (R1), retention time (R2) and were considered as response factors (dependent factors). The significance of independent factors was determined using Fisher's statistical test for Analysis of the Variance (ANOVA) model that was estimated. C-18 column (150 mm × 4.6 mm, 5 µm pore size), column was the most suitable one since it produced symmetrical peaks with better resolution. The UV detector response of melatonin was studied and the best wavelength was found to be 222 nm showing highest sensitivity of both compounds. The method was validated for specificity, reproducibility, accuracy, linearity, robustness and solution stability and can be used for the assessment of quality of drug product in development and stability samples of the marketed formulation. The target degradation for the stability indicating ability of the assay method was tried in the present study and there was no any interfering peaks found due to degradation products.

INTRODUCTION

Analytical methods play an important role supporting implementation of QbD in process Pharmaceutical development and development and manufacturing. Analytical testing also plays prominent role in Pharmaceutical development, risk assessment, process monitoring and control and continuous quality assessment throughout the product. Quality-by-Design (QbD) is well-established in development and manufacture of pharmaceutical drug substance and drug product and is discussed in ICH Q8, [1] Q9 and Q2. The same QbD approach can be applied to analytical procedures as per ICH Q2. In addition, there is now a technique to definitively link the data to its intended use. These are exciting times for testing laboratories and the users of the data they produce. The knowledge obtained during development helps to justify the establishment of a design space, (process) control strategy and set point within the (regulatory approved) design space. Materials made within the design space will produce an acceptable product, and changes within the design space are regulatory acceptable. Quality by Design approach suggests looking into the quality of analytical process during the development stage itself. It says that quality should be built into the process design rather than testing into final results of analytical process. QbD is defined as ―a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management. In alignment with the approach proposed in the draft FDA guidance for process validation, a three-stage approach can be applied to method validation [2-3].

Stage 1. Method Design: Define method requirements and conditions and identify critical controls.

Stage 2. Method Qualification: Confirm that the method is capable of meeting its design intent. Stage 3. Continued Method Verification: Gain ongoing assurance to ensure that the method remains in a state of control during routine use. A critical function of Stage 1 is the design of an Analytical Target Profile (ATP) for the method. To design the ATP, it is necessary to determine the characteristics that will be indicators of method performance for its intended use. These are selected from the performance characteristics described in ICH Q2 as per the traditional approach. Instead of being applied in a tick box manner, they are

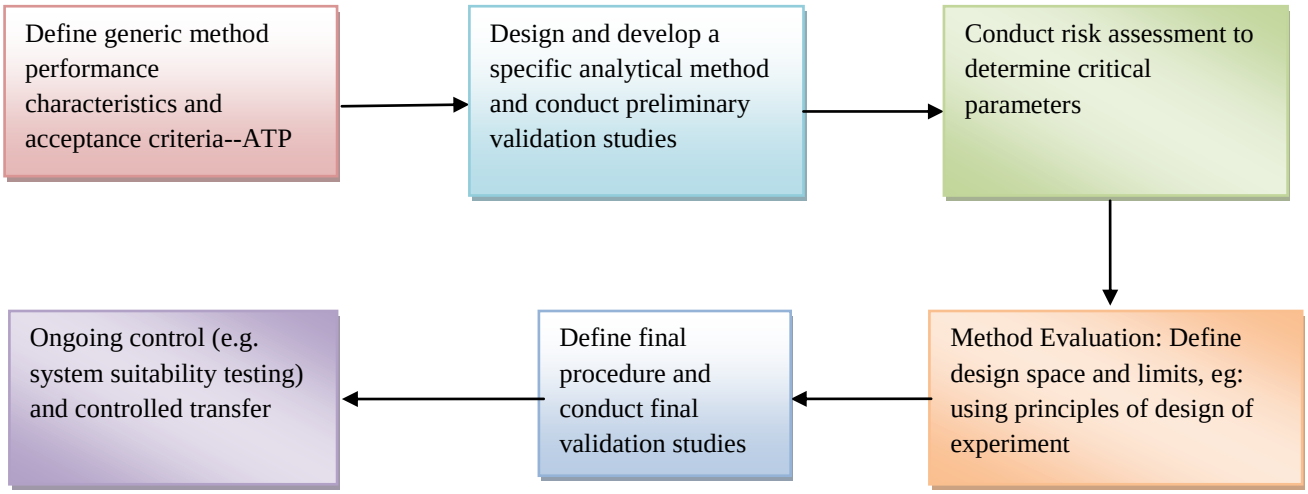

Fig 1-QbD work flow

Investigated by a risk assessment exercise as described in ICH Q9 in combination with carefully designed development studies to identify the critical method and sources of variation. Variables are then investigated by robustness and ruggedness experiments to understand the functional relationship between method input variables and each of the method performance characteristics and the results are compared to the desired outcome defined in the ATP. From this, one can identify a set of operational method controls. Also, having evaluated the critical method parameters and gained a better understanding of the method through structured experimentation [3]. Addition to validating the method characteristics as per regulatory guidance, verifying the accuracy and precision provides additional understanding of the method's measurement uncertainty and confirms conformance to the previously defined method performance requirements (ATP). This can be accomplished through a joint accuracy and precision.

Melatonin (N-acetyl-3-(2-aminoethyl)-5-methoxyindole), an endogenous hormone, is a predominant product of the pineal gland. It is secreted in a rhythm that is strictly dependent on the light–dark cycle. The melatonin plasma concentration rises in the early night, peaks at about midnight and then declines during the daytime [4]. Melatonin also has an age-related rhythm. The reduction of melatonin contributes to the aging process and may shorten the life span. Studies have shown that exogenous melatonin administration is useful for treating circadian disruptions, e.g. insomnia, jet lag Moreover; melatonin has pharmacological effects on the treatment of Alzheimer's disease, Parkinson disease, glaucoma, depressive disorder, breast and prostate cancer, hepatoma and melanoma. Unfortunately, a substitution therapy is not easily achieved with melatonin because of its relatively poor bioavailability and rapid

elimination.

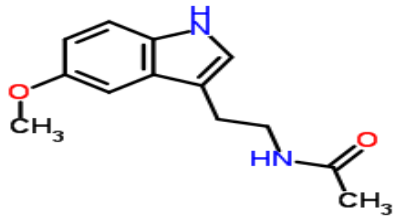

Fig. 2 Melatonin

In patients with sleep disorders and altered circadian rhythms, such as occur in jet lag, night shift work, and various neuropsychiatric disorders, oral administration of melatonin can provide the necessary resynchronization of those cycles, at dosages ranging from 0.3 to 8 mg. Synthesis of melatonin from the amino acid tryptophan is decreased by exposure to magnetic fields and by the aging process. Melatonin is a potent scavenger of free radicals and exerts direct inhibition of cancer growth. Various cancer types have been shown to be responsive to oral melatonin (10-50 mg daily), including breast cancer, non-small-cell lung cancer, metastatic renal cell carcinoma, hepatocellular carcinoma, and brain metastases [5] from solid tumors. Melatonin has also been reported to lower LDL- and total cholesterol levels. Abnormally low melatonin levels have been theorized to be a factor in multiple sclerosis, coronary heart disease, epilepsy, and postmenopausal osteoporosis. These reports, while preliminary, serve to further illustrate the wide range of potential effects exerted by melatonin [6-8]. It is soluble in water; soluble in methanol, ethanol, slightly soluble in alcohol and in chloroform; and very slightly soluble in acetone.

The present work aims at systematic development of a simple, rapid and highly sensitive method for the analysis of Melatonin by QbD approach.

MATERIALS AND METHOD

Table No: 1 -Drug Name

Drug	Melatonin
Source of drug	Otto chemicals

Chemicals:

Table No: 2 - Reagent and chemicals

Sr. No.	Name of reagent used	Make
1.	Water	Analytical and HPLC grade
2.	Methanol	Analytical and HPLC grade
3.	Acetonitrile	Analytical and HPLC grade
4.	Ethanol	Analytical grade

Instruments:

Table No: 3 - List of instruments

Sr. No.	Name of Equipment	Source
1.	HPLC	Agilent 1220 Infinity LC
2.	UV	Shimadzu, Model: UV-1800
3.	Detector	Variable wavelength detector
4.	Electronic weighing balance	Shimadzu BL- 220 H
5.	Hot air oven	Nisco company
6.	Sonicator	The ultrasonics PCi Analytics sonicator

Table. No. 4 - Chromatographic Conditions

Sr. No.	Give title	Chromatographic Conditions
1.	Concentration	10 µg/ml
2.	Column	Inertsil ODS 3V-(GL Sciences Inc.) 4.6×250 mm, 5 µm
3	Mobile phase	Methanol
4.	Wavelength	222 nm
5.	Flow rate	1 ml/min
6.	Run time	5 min
7.	Injection time	20 µl

DRUG AUTHENTIFICATION

1. Melting point (M.P)

Sample obtained was characterized for melting point of the substance. The melting point was determined by introducing small amount of substance in capillary and constant heat was applied. The drug substance was tested in the temperature range of 116~118ºC and the melting point were noted.

2. Solubility

The solubility of drug sample was tested in various solvents like Soluble in methanol, water (0.1 mg/ml), ethanol (8 mg/ml), benzene, chloroform, the observed results were then compared with drug profile.

B. Solution Preparation Method:

i. Standard stock solutions:

A stock solution of 100 µg/ml of Melatonin was prepared by dissolving 10 mg melatonin in 100 ml methanol. Standard calibration solutions were prepared by dilution of the stock solutions using the diluent. These solutions were considered at seven different levels which were 2 µg/ml,4 µg/ml,6 µg/ml,8 µg/ml,10 µg/ml,12 µg/ml were prepared in diluent the calibration curves for melatonin was constructed by plotting the peak area against the drug concentration. The diluent was methanol (100 v/v).

ii. Selection of detection wavelength:

The detection wavelength was selected by scanning the 10 µg/ml concentration solution of melatonin in the mobile phase in UV spectrophotometer and maximum absorption was selected as 222 nm.

iii. Selection of mobile phase:

The pure drug of Melatonin was injected into the HPLC system and run in different mobile phase system. Different mobile phases like acetonitrile, methanol, water, and different pH buffer were tried. It was concluded that methanol gives satisfactory results which pass the ICH guideline i.e. ICH Q2 (R1). Hence finalized mobile phase is Methanol.

iv. System suitability test (SST)/ Specificity and formulation analysis:

The specificity of method was established by preparing placebo solution by an optimized method for assay of the samples using equivalent weight of the placebo with marketed preparation. Chromatogram of the placebo was not showing any interference at the retention time of melatonin. The SST ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters tested met the acceptance criteria on all days. The system suitability assessment for the analytical HPLC method established instrument performance parameters such as peak area, % R.S.D., column efficiency (N) and USP tailing factor (Tf) for both the analytes.

v. Analytical target profile

"QbD is systematic approach to product, process design and development". Hence it began with determination of goal or method intent. Here method intent was to develop HPLC method of melatonin which is robust, accurate, precise and USP tailing less than 2, number of theoretical plates as per requirement and short analysis time i.e. less than 10 min. as per QbD norms, a robust method should be developed with help of visualized a design space.

vi. Formulation analysis and system suitability/ Specificity

The assay for the marketed tablet was established with presents chromatographic condition developed and it was found to be more accurate and reliable. The average drug content was found to be 100.9 % for RIS, 100 % for BA. With % RSD for melatonin was 0.1 and 0.1 %.

vii. Linearity and range:

Linearity was determined for Melatonin in the range of 2 µg/ml to12 µg/ml of test solution concentration. The correlation coefficient ('r2') values were 0.999 (n = 6) indicating an excellent correlation between peak areas and analyte concentrations.

viii. Accuracy (% Recovery)

The mean percentage recoveries obtained were 101.3%, 100% for Melatonin. The developed method was found to be accurate as the mean percentage recoveries obtained for Melatonin was found to be within limit as recommended by ICH guidelines. The developed method was

found to be accurate as the % RSD values for accuracy studies was <2%, as recommended by ICH guidelines.

ix. Method Precision:

The system precision was demonstrated by preparing the standard solution at test concentration and injected repeatedly for six times. The % RSD for repeatability of sample preparation is 0.12 % and 0.07 % for Melatonin. The precision is satisfactory and the % RSD is not more than 2.0% as per ICH guideline.

x. Robustness and solution stability studies:

The % Assay and % RSD was found to be in range 100 ± 1.5% and <2, respectively. It indicates that method follows specification of ICH guideline. Results of the stability studies were in the range of 99.5 - 101.5%. Stability, as described in method development under experimental section, was studied. Results of the stability studies were within the acceptable limit (98 - 102%).

5.3.3. Chromatographic Method development by QbD approach [20]

1. Define method intent

The goals of HPLC method development have to be clearly defined, as pharmaceutical QbD is a systemic, scientific, risk based, holistic and proactive approach that begins with predefined objectives and emphasizes product and process understanding and control.

2. Perform experimental design

A systematic experimental design is needed to assist with obtaining in-depth method understanding and performing optimization. Here an efficient and comprehensive experimental design based on systematic scouting of two key components of the RP-HPLC method (mobile phase and pH) was presented. It forms a chromatographic database that will assist with method understanding, optimization and selection. In addition, it can be used to evaluate and implement change of the method, should it be needed in the future, for example, should the chromatographic column used no longer be commercially available, or an impurity is no longer relevant.

3. Factorial Design

Central composite statistical screening design was used to optimize and evaluate main effects, interaction effects and quadratic effects of the formulation ingredients on the in-vitro release of the drug. A 2-factor, 2-level design used is suitable for exploring quadratic response surfaces and constructing second order polynomial models with Design Expert 9 (Version 9.0.5.1) Stat-Ease. Inc, 2021 East Hennepin Ave, Suite 480, Minneapolis, MN 55413. $Y = \beta0 + \beta1A + \beta2B + \beta12AB + \beta11A2 + \beta22B2$

Where Y is the measured response associated with each factor level combination; β0 is an intercept; β1 to β22 are regression coefficients computed from the observed experimental values of Y from experimental runs; and A and B are the coded levels of independent variables. The terms AB, A2 and B2 represent the interaction and quadratic terms, respectively. The factors were selected based on preliminary study. Flow rate (A) and wavelength (B) were selected as independent variables. The Retention time and peak area were selected as dependent variables.

5.3.4. Forced Degradation [47]

a. Acid degradation:

The preparation of 0.01 N hydrochloric acid (HCl) was done by diluting 0.085 ml of conc. HCl to 100 ml of distilled water as mentioned in IP. Melatonin was accurately weighed and transferred to a labeled round bottomed flask and 10 ml 0.01 N hydrochloric acid (HCl) was added. Reflux the sample for 2 hrs. Further, make the dilutions to get final concentration 10 µg/ml. Measure the absorbance in UV at 222 λ max.

10 mg drug + 0.01 N HCl (10ml) = reflux 2 hrs = Further make the dilutions to get final concentration 10 µg/ml

b. Base degradation:

The preparation of 0.01 N Sodium Hydroxide (NaOH) was done by dissolving 0.04 gm of sodium hydroxide pellets in 100 ml of distilled water as mentioned in IP. Melatonin was accurately weighed and was transferred to a labeled round bottomed flask and 10 ml 0.01 N

Sodium Hydroxide (NaOH) was added. Reflux the sample for 2 hrs. Further, make the dilutions to get final concentration 10 µg/ml. Measure the absorbance in UV at 222 λ max.

10 mg drug + 0.01 N NaOH (10 ml) = reflux 2 hrs = Further make the dilutions to get final concentration 10µg/ml

c. Neutral condition:

10 mg drug was weighed and transferred to 10 ml water in round bottom flask. Refluxed for 2 hours. Further, the dilutions were made to get final concentration 10 µg/ml. Absorbance was measured in UV at 222 λ max.

10 mg drug + Dist. Water (10 ml) = reflux 2 hrs = Further make the dilutions to get final concentration 10 µg/ml

d. Photo stability study:

Photo stability was performed by placing 10 mg of melatonin in daylight for 24 hours. The samples were diluted with mobile phase up to 100 ml in a volumetric flask. 1 ml sample diluted up to 10 ml by mobile phase. Absorbance was measured in UV at 222 λ max.

e. Dry heat:

Standard quantity of melatonin was placed in an oven at 6^0C for 2 hours to observe degradation behavior of drug. To make stock solution (100 ppm) 10 mg drug was taken and diluted up to 100 ml in a volumetric flask with mobile phase. 1 ml was pipette out and was diluted up to 10 ml by mobile phase. Absorbance was measured in UV at 222 λ max.

f. Hydrogen peroxide degradation:

10 mg drug was weighed accurately and transferred into 10 ml (10%) H_2O_2 in round bottom flask. These samples were kept in a clean baker in a dark room for 2 hours. Further dilutions were made to get final concentration 10 µg/ml. Absorbance was measured in UV at 222 λ max.

10 mg drug + (10%) H_2O_2 10 ml = were kept in a clean baker in a dark room for 2 hours.= Further make the dilutions to get final concentration 10 µg/ml.

Design of Experiment:

Table.No.5: 2^2 Factorial design with upper and lower limits of all factors Statistical Optimization technique

2 Factors	2 Levels	
	Low	**High**
Flow Rate	0.8 ml / min	1.2 ml /min
Wavelength	220 nm	224 nm

The optimization phase was designed statistically using 2^2 factorial design in which two variables namely Flow rate, wavelength was kept at two levels that is low, and high. Main interactive influences were tested using statistical methods. The twenty trials of optimization phase were estimated. Although all trials were analyzed for Rt of all of these parameters were considered for selection of best chromatographic condition in the optimization phase.

Table. No. 6: 2^2 Factorial design with upper and lower limits factors Statistical sequence for optimization

Run No.	Replicates	Flow Rate	Wavelength	Rt
1	6	1.2	222	2.4
2	13	1	222	2.89
3	4	0.8	222	3.69
4	3	1.2	220	2.44
5	1	0.8	220	3.69
6	5	1	222	2.89
7	12	1	222	2.89
8	9	1.2	224	2.94
9	8	1	224	3.1
10	2	1	220	2.6
11	11	1	222	2.89
12	7	0.8	224	2.9
13	10	1	222	2.89

Statistical analysis of the data and optimization

Polynomial models including linear, interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA). The best fitting model was selected based on the comparisons of several statistical parameters including the coefficient of variation (CV), the coefficient of determination (R^2), adjusted coefficient of determination (adjusted R^2) and the predicted residual sum of square (PRESS) provided by the Design Expert software. In addition, statistical analysis namely the analysis of variance (ANOVA) to identify the significant effect of factors on response, regression coefficients, F test and P value were also calculated with the software.

The relationship between the dependent and independent variables was further elucidated by using contour and response surface plots (Figure 17-21). These plots are useful in the study of the effects of factors on the response at one time and predict the responses of dependent variables at the intermediate levels of independent variables. Subsequently, a numerical optimization technique by the desirability approach (Figure 20) and graphical optimization technique by the overlay plot (Figure 21). To validate the chosen experimental design, the resultant experimental values of the responses were quantitatively compared with those of predicted values and the percentage relative error was calculated by the following equation:

% Relative error = Predicted Value – Experiment Value/ Predicted Value × 100

Data analysis

The model parameters obtained from the analysis of variance (ANOVA) for the response of drug are shown in tables IV-VII. These parameters were used to construct the models that describe the effect of the independent variables on the responses.

The experimental design was prepared to obtain drug was evaluated for their retention time. The F values for the responses retention time found to be 11.74, which indicate that the models are significant. The values of Prob>F less than 0.05 for all the responses are indicating that the models are significant. The response of model terms A, B, AB, for Retention time was found to be significant. The F value of lack of fit for retention time was found to be 11.74 which implies that the lack of fit is significant. Similarly ‘R- squared’ value was also calculated for all responses and found to be closer to the ideal value (i.e. zero). High ‘R- squared’ value signifies that the model terms are highly correlated to each other leading

to a poor model. In contrast to this 'R- squared' value obtained in the present model is close to zero, which indicates a good model. In all the cases 'Pred R squared' values are in reasonable agreement with the 'Adj R squared' values. In all the cases 'Adeq Precision' values are in the range of 0.2296– 13.066 indicating an adequate signal and that the model can be used to navigate the design space. The VIF (variance inflation factor) values for all models were found to be near to one indicating a good estimation of coefficient. The application of response surface methodology yielded the following regression equations which give an empirical relationship between the logarithmic values of retention time. Test variables in coded units:

$$Rt = 2.93 - 0.42*A + 0.035*B + 0.32*AB$$

Table.No.7 ANOVA for Response Surface 2FI model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	Remark
Model	1.47	3	0.49	11.74	0.0018	Significant
A-Flow rate	*1.04*	*1*	*1.04*	*25.04*	*0.0007*	
B-Wavelength	*7.350E-003*	*1*	*7.350E-003*	*0.18*	*0.6841*	
AB	*0.42*	*1*	*0.42*	*10.00*	*0.0115*	
Residual	0.37	9	0.042			
Lack of Fit	*0.37*	*5*	*0.073*	*32.78*	*0.0024*	*Significant*
Pure Error	*8.920E-003*	*4*	*2.230E-003*			
Cor Total	1.84	12				

Std. Dev.	0.20	R-Squared	0.7964
Mean	2.93	Adj R-Squared	0.7286
C.V. %	6.95	Pred R-Squared	0.2296
PRESS	1.42	Adeq Precision	13.066

Table. No 8 Model of coefficients (model of regression coefficients)

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.93	1	0.057	2.81	3.06	
A-Flow rate	-0.42	1	0.083	-0.61	-0.23	1.00
B-Wavelength	0.035	1	0.083	-0.15	0.22	1.00
AB	0.32	1	0.10	0.092	0.55	1.00

Externally Studentized Residual

The model that has been developed can be used to predict the resolution of melatonin within the limits of the experiments.The normal probability plot of the residuals versus the predicted response and the residuals versus the actual response is shown in Figs. 3 and 4. Close inspection of Fig. 3 reveals that the residuals generally fall on a straight line which indicates that the errors are normally distributed, thus supporting the fact that the model fits the data adequately. These plots are very important and are required to check the normality assumption in a fitted model.

Residual Vs Predicted

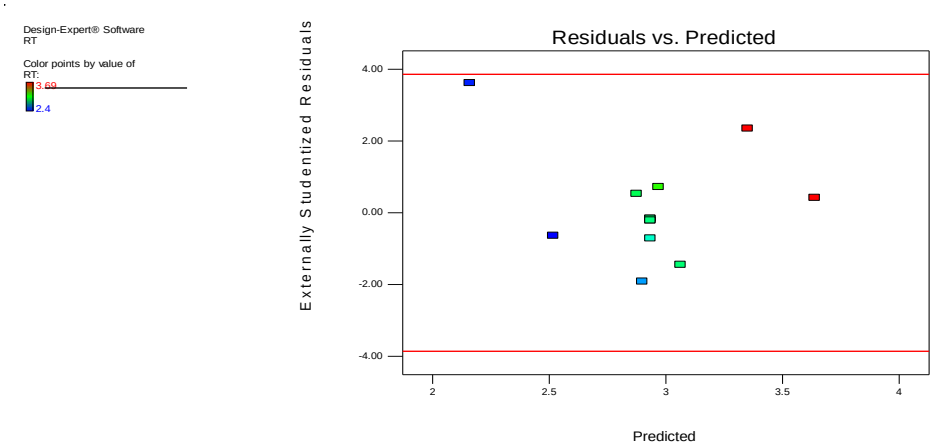

Figure 3: Externally studentized residual graph of Residual Vs Predicted

Residual Vs Actual

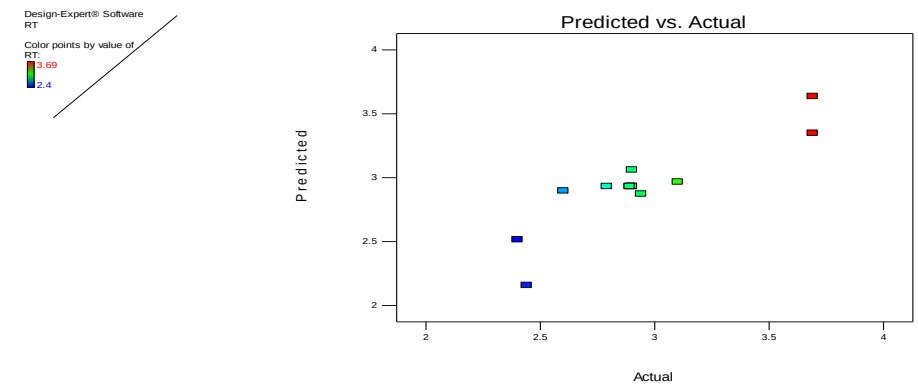

Figure 4: Externally studentized residual graph of Residual Vs Actual

Response surface methodology in 3D and 2D (Counter plot)

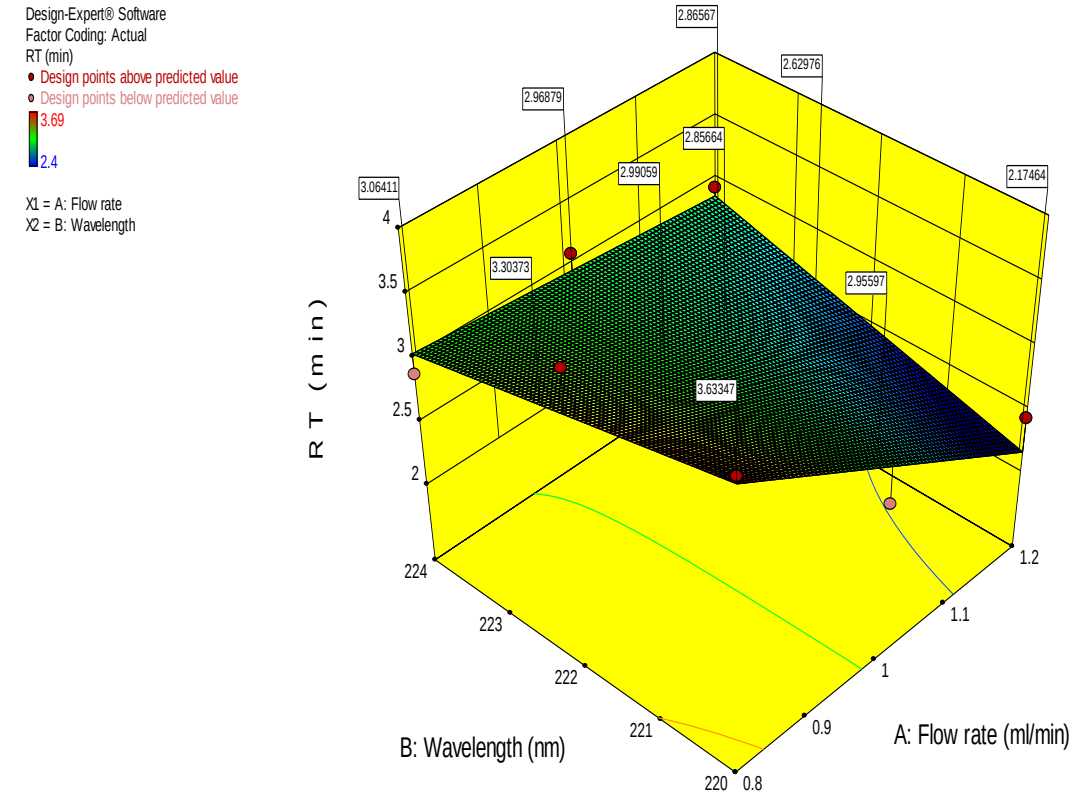

Figure 5: Response surface diagram showing combined effect of flow rate and wavelength on Rt of compounds at low level and high level

Contour plots:

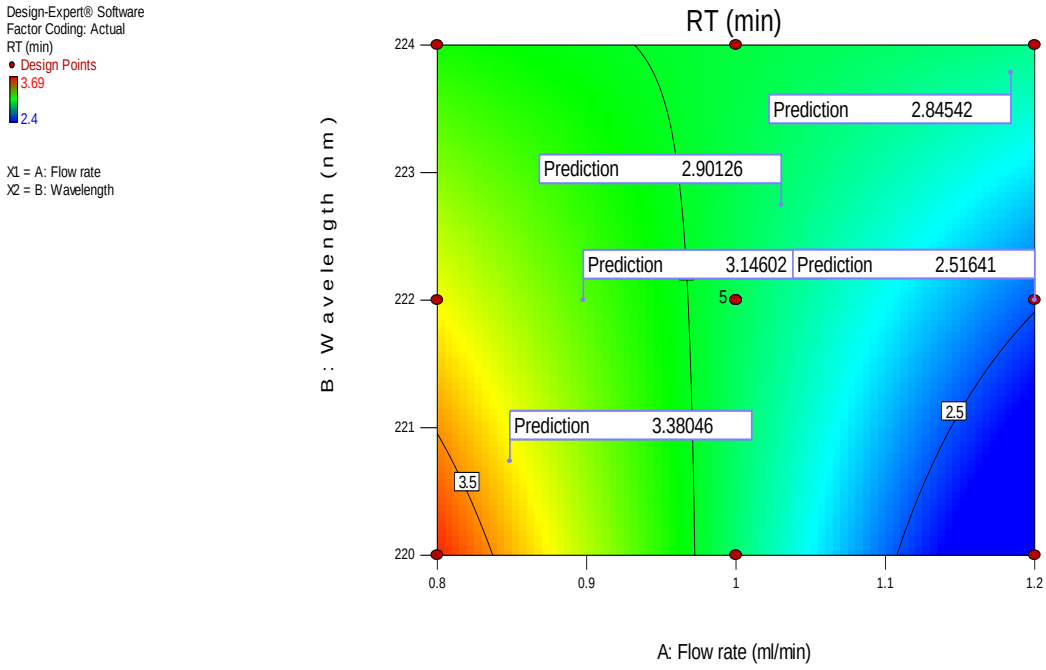

Figure 6: Contour plot showing effect of flow rate and wavelength on Rt of compounds at low level

Contour plots:

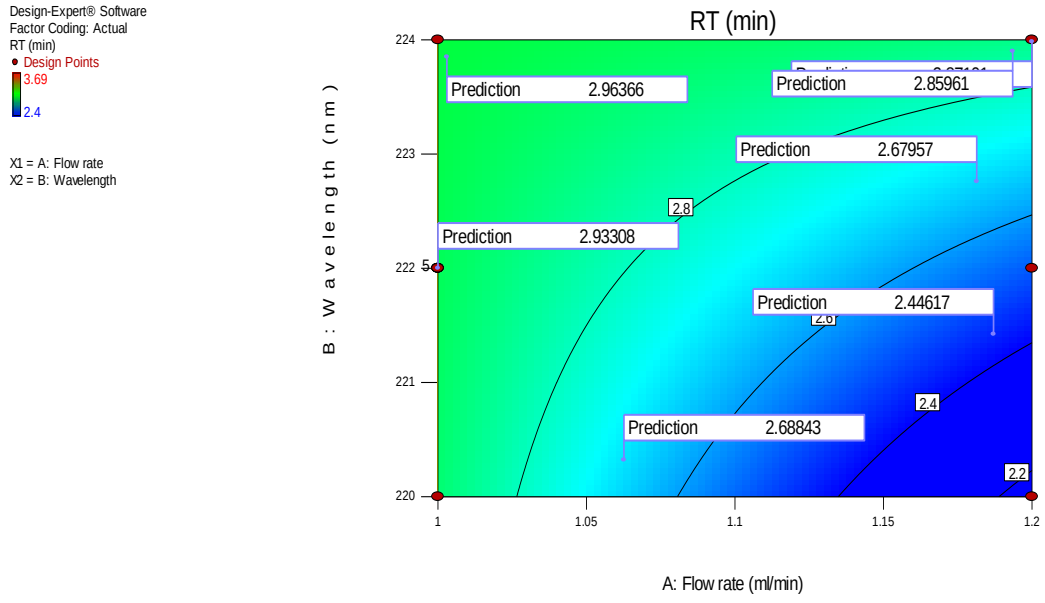

Figure 7: Contour plot showing effect of flow rate and wavelength on Rt of compounds at high level

Desirability

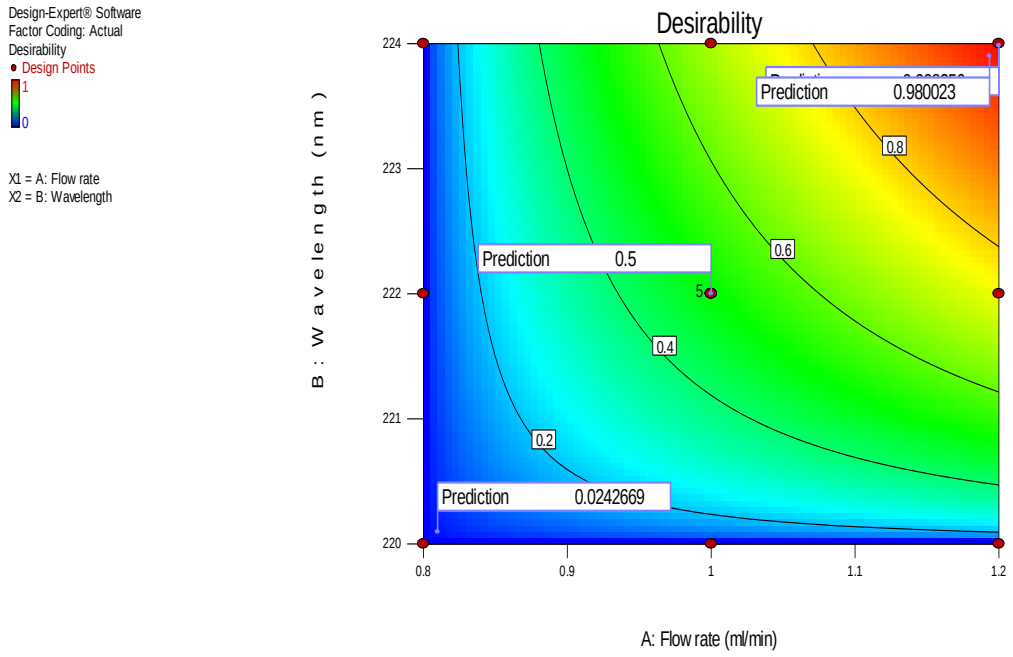

Figure 8: Desirability

Overlay Plot of Design point

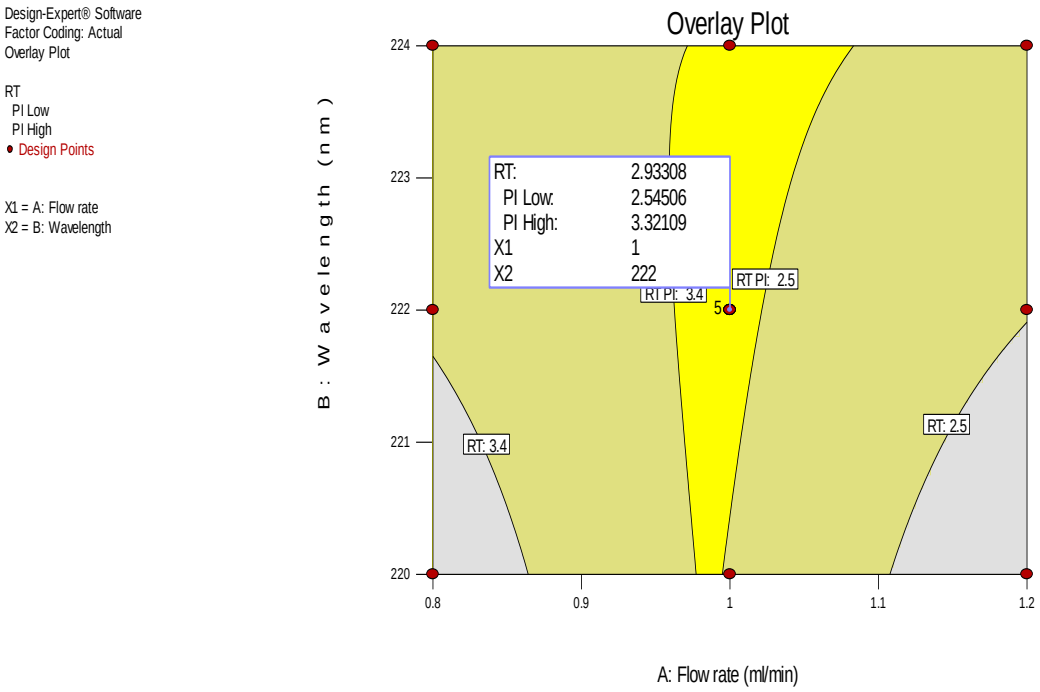

Figure 9: Overlay Plot of Design point

Flow rate, wavelength were significant by linear regression. Two dimensional **Contour** plots and response surface plots are presented in Figs. 6–7 and are very useful for studying the interaction effects of the factors on the Rt. Rt of melatonin increases as the wavelength increases. (Fig.8 and 9). Fig. 23 shows that increase in wavelength increases the Rt and increase in flow rate decreases the Rt. The effect of wavelength and flow rate on the Rt of melatonin was investigated; as can be seen from the contour plots (Figs. 5 – 7), it has no effect on Rt. The effect of flow rate on the Rt of melatonin was investigated in a range of 2.7 – 2.9.

The Standard Chromatogram- 10 µg/ml

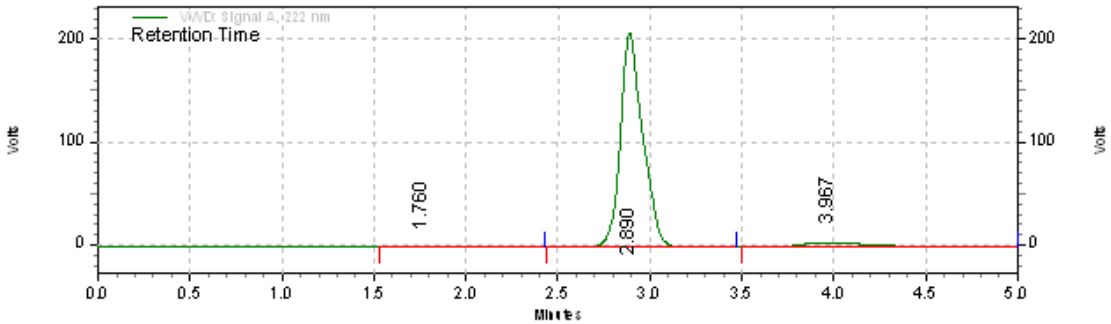

Fig. 10- Standard chromatogram

Linearity

Table No 9–linearity data

Sr. No.	Concentration (µg/ml)	Area
1	2	5954883
2	4	11834974
3	6	17457656
4	8	23202095
5	10	29419190
6	12	34567029

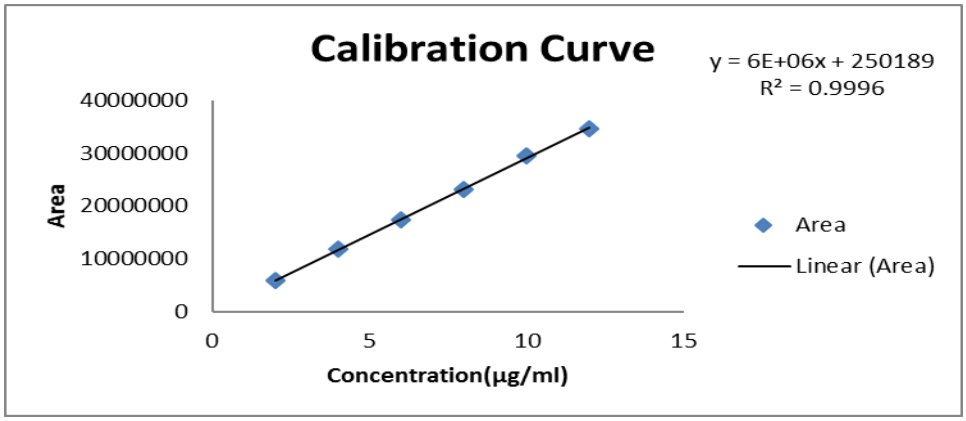

Fig. 11-Calibration curve

Table No 10 - Linearity parameter

Parameter	HPLC method
Range	2-12 µg/ml
Correlation coefficient	0.999
Slope	6E+06X

Accuracy

Table No 11–Accuracy Data

Accuracy level	% recovery	AVG	Statistical analysis		
			mean	SD	% RSD
80 % 1	99.20	97.17%	22611382	510216.5	0.22
80 % 2	86.36				
80 % 3	105.95				
100 % 1	97.78	99.99%	27970345	1706857.5	0.610
100 % 2	101.89				
100 % 3	100.46				
100 % 4	101.83				
100 % 5	96.08				
100 % 6	101.93				
120% 1	102.68	101.77%	33920096	1188979.3	0.350
120% 2	101.25				
120% 3	101.40				

Precision

Table No 12-Intraday precision Data

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	2.963	29455609
2.	10	2.893	28748612
3.	10	2.913	25960047
4.	10	2.910	25645377
5.	10	2.917	28721368
6.	10	2.927	29291062
AVG		2.92	27970345
SD		0.023594	1706857.5
%RSD		0.80	0.610

Table No 13 – Inter day precision data

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	2.900	27066585
2.	10	2.903	28878334
3.	10	2.907	28991479
4.	10	2.943	29533207
5.	10	2.900	28137788
6.	10	2.890	29270779
AVG		2.90	28646362
SD		0.018433	905941.53
% RSD		0.0063	0.3162

Robustness

Table No 14 - Change in the flow rate 0.8 ml/ml

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	3.690	31716575
2.	10	3.683	30237311
3.	10	3.677	31141603
4.	10	3.683	32651316
5.	10	3.680	29088182
6.	10	3.687	31486443
AVG		3.68	31053571
SD		0.004676	1242298.3
% RSD		0.12	0.400

Table No 15 - Change in the flow rate 1.2 ml/ml

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	2.447	18114397
2.	10	2.447	21694852
3.	10	2.447	18117729
4.	10	2.443	18386884
5.	10	2.447	18584694
6.	10	2.443	18188195
AVG		2.44	18847791.833
SD		0.002066	1406651
% RSD		0.084	0.746

Table No. 16-Change in the wavelength at 220 nm

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	2.940	26717509
2.	10	2.940	24548753
3.	10	2.937	23387723
4.	10	2.943	23101552
5.	10	2.940	22731452
6.	10	2.943	22682375
AVG		2.94	23861460
SD		0.0022583	1555534.2
% RSD		0.076	0.651

Table No. 17-Change in the wavelength at 224 nm

Sr. No.	Concentration (µg/ml)	Rt	Area
1.	10	2.940	26014683
2.	10	2.937	22307251
3.	10	2.950	26377531
4.	10	2.940	22724890
5.	10	2.940	21518118
6.	10	2.940	24637242
AVG		2.94	23929952
SD		0.004491	2036628.7
% RSD		0.17	0.81

Limit of Detection and Limit of Quantification

$$LOD = \frac{3.3\sigma}{S} \quad LOQ = \frac{10\sigma}{S}$$

Where,

σ = standard deviation

S = slope of calibration curve

Table No18–LOD and LOQ data

LOD	5.92
LOQ	17.95

Analysis of Marketed Formulation

The peak at 2.913 was observed for melatonin in the chromatogram of the drug sample extracted from tablets. Experimental results of the amount of melatonin in tablets, expressed as percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The drug content was found to be 99.15% and % RSD found to be is 0.057 for melatonin in tablet form.

Table No 19 –Analysis of Marketed Formulation

Sr. No.	**Concentration (µg/ml)**	**area**	**% accuracy**
1.	10	29359190	
2.	10	29358192	
3.	10	29378889	
4.	10	29368791	99.15%
5.	10	29368792	
6.	10	29403696	
AVG		29372925	
SD		16865.983	
% RSD		0.057	

ASSAY-

The % purity of the melatonin tablet was found to be 99.15% purity.

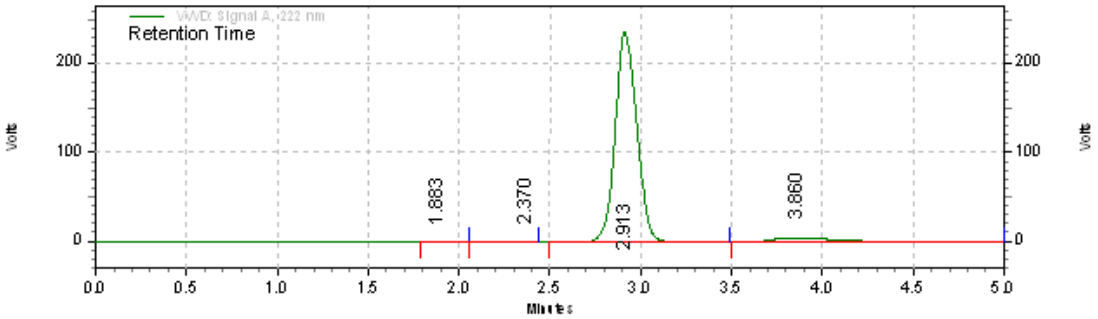

Fig 12- Chromatogram for Assay

Force Degradation for 2 Hours

Table no 20– Short term force degradation data

Stress condition	% Degradation	Remark
0.01N NaOH	95%	Unstable
0.01N HCl	71%	Unstable
Oven	72%	Unstable
Water	17%	Stable

Degradation Chromatogram

0.01 N NaOH

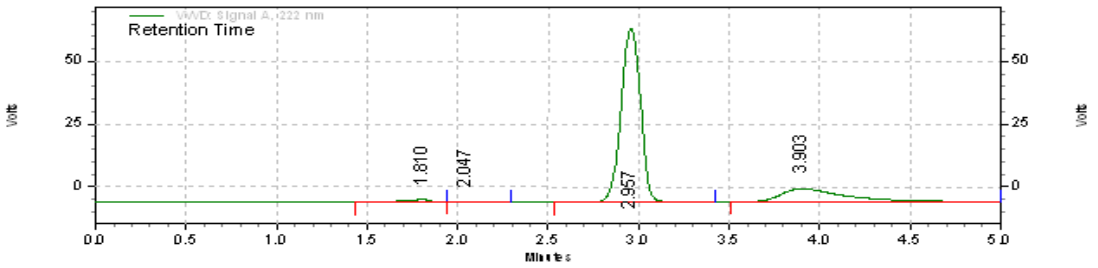

Fig No 13- Chromatogram for 0.01 N NaOH

0.01 N HCl

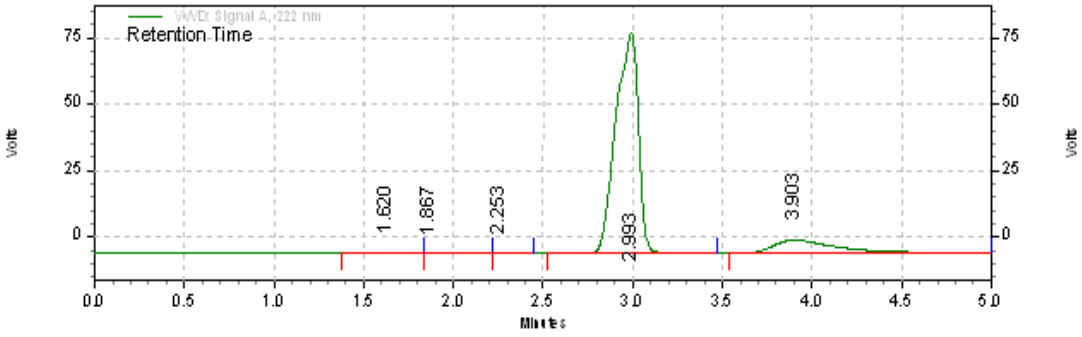

Fig No 14- Chromatogram for 0.01 N HCl

Oven (60°C)

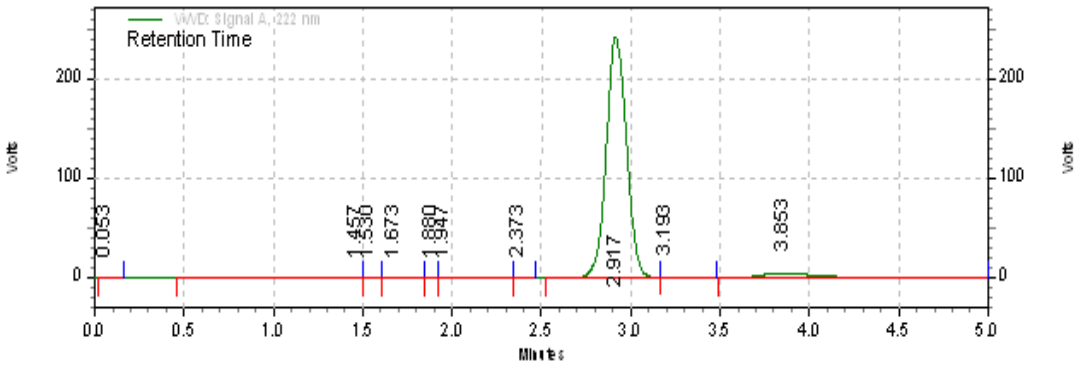

Fig No 15- Chromatogram for Oven

Water

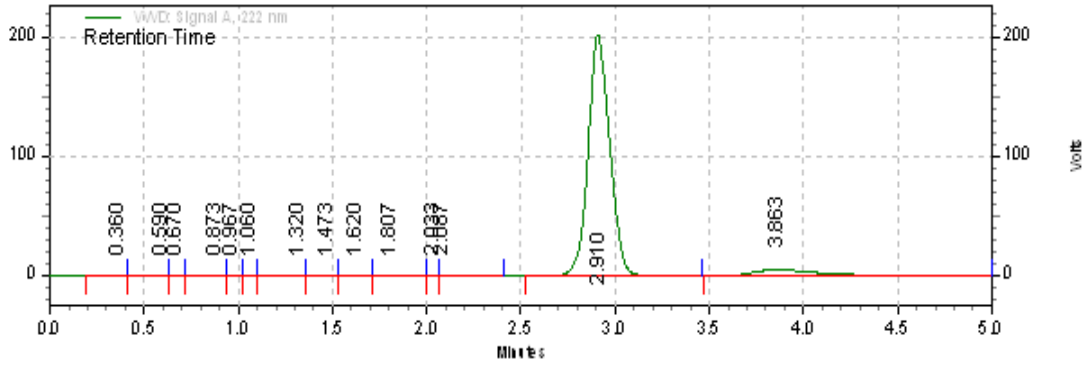

Fig No 16- Chromatogram for Water

CONCLUSION

A robust method for degradation of melatonin was developed using a Quality by Design approach on a Design-Expert® Software, Version 9. Three independent factors were used such as flow rate (A), wavelength (B). Totally 13 experimental runs were suggested by the software for analyzing the interaction of each level on formulation characters and the peak area (R1), retention time (R2) were considered as response factors (dependent factors). The method was validated according to ICH guidelines. Specificity of the method was determined by analyzing samples containing a mixture of the drug product and excipients. The assay for the marketed oral solution was established with present chromatographic condition developed and it was found to be more accurate and reliable. The % Assay and % RSD was found to be in range 100 ± 1.5% and <2, respectively. It indicates that method follows specification of ICH guideline. Results of the stability studies were in the range of 99.5 - 101.5%. As per ICH guidelines, the target degradation for the stability indicating ability of the assay method was tried in the present study. No interfering peaks were found due to degradation products at the drugs Rt's. Design Expert was able to automatically predict and test speed and resolution optimized analytical methods that separated all the drug peaks. Analytical to prep scale-up of the drug peaks was successful with sufficient resolution of the critical peak pairs to ensure that maximum recovery of pure fractions would be possible.

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