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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BISOPROLOL FUMARATE BY QBD APPROACH FORM

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ABSTRACT

A new approach to drug development could increase efficiencies, provide regulatory relief, flexibility, and offer important business benefits throughout the product's life cycle. Quality by design is a systemic approach and essential part of the modern approach for quality and pharmaceutical development. It includes defining target product quality profile, designing product, developing formulations, manufacturing processes, identifying critical quality attributes, process parameters, sources of variability and controlling manufacturing processes to ensure consistent product quality over time. Two independent factors were used such as flow rate (A), wavelength (B). Totally 27 experimental runs were suggested by the software for analyzing the interaction of each level on formulation characters and the peak area (R1), tailing factor (5%) (R2) and number of theoretical plates USP (NTP) (R3) 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. Waters cosmosil 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 wavelength was found to be 233 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 oral tablet. 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

KEY WORDS

QbD, (ANOVA), target degradation, life cycle, Quality Attribute, product life cycle

INTRODUCTION

Bisoprolol is a drug belonging to the group of beta blockers, a class of drugs used primarily in cardiovascular diseases. More specifically, it is a selective type β_1 adrenergic receptor blocker. It is used for secondary prevention of myocardial infarction (MI), heart failure, angina pectoris and mild to moderate hypertension. Bisoprolol is structurally similar to Metoprolol, Acebutolol and Atenolol in that it has two substituents in the para position of the benzene ring which might be the reason for its β1-selectivity. At lower doses of less than 20 mg a day, Bisoprolol selectively blocks cardiac β1-adrenergic receptors with very less action against β_2-adrenergic receptors of the lungs and vascular smooth muscle. Bisoprolol is beneficial in treatment for: hypertension, cardiac ischemia and congestive heart failure. This is also used for preventative treatment before and primary treatment after heart attacks thus reducing the chances of recurrence.1 During hypertension there is an elevated blood pressure, which is what bisoprolol targets.2,3 While in cardiac ischemia the drug is used to reduce the activity of the heart muscle and therefore reduce oxygen and nutrient demand, so reduced blood supply can still transport sufficient amounts of oxygen and nutrients.4,5,6

There a very less or no detailed literature published on how to implement AQbD approach in method life cycle. Nevertheless, this approach is very widely known in development/optimization of pharmaceutical formulation or processes. Whenever an analyst thinks to apply DOE, he may have a misperception that the model uses more trials or practically it is difficult to select a design or interpret data Quality is a heart of the pharmaceutical industry. The ICH Q8Q11 is described the concepts of Analytical Quality by design (QbD) for drug and related substance. The ICH Q8 is Pharmaceutical Development, ICH Q9 is Quality Risk Management, and ICH Q10 is Pharmaceutical Quality Systems is the ICH guidelines gives the complete procedure for applying analytical QbD process. QbD technique particularly used for developing the analytical method for selected drugs to identifying and quantifying the active content and minimizing source of variability. The applications of multivariate statistical techniques for the optimization of chromatographic and Spectroscopic systems. The surface response methodologies, central composite design, Doehlert matrix, and Box– Behnken design systems. For QbD. A very useful component of QbD is the understanding of factors and their interaction effects by a desired set of experiments. For the purpose of QbD for HPLC methods, robustness and ruggedness should be verified early in the method development stage to ensure method performance over the lifetime of the product. Quality by design principles are applied to build in a more scientific and risk based multi factorial approach to the development and validation of analytical methods using HPLC and spectroscopic technique**.** [3] given a due consideration. Some of the parameters include molecular properties (physical and chemical) of analyte, its related impurities, cost of reagents, sensitivity and selectivity of method, availability of instrument, time required for analysis. For a molecule with significant Ultra violet (UV) chromophore Liquid Chromatography (LC) with UV detector is appropriate method of detection but in case of no chromophore Gas Chromatography consideration may be given to other chromatographic methods or derivatization methods. In addition, presence of ionizable centers, polarity of molecule should be reread. Method design in terms of AQbD includes space generation, defining Analytical Target Profile (ATP), experimental design screening and establishing Critical Quality Attributes (CQA's).

High-performance liquid chromatography (HPLC) (8-12), particularly Reversed Phase HPLC (RP HPLC), is the most popular analytical technique in the pharmaceutical industry. The quality of HPLC methods has become increasingly important in a QbD environment. For the purpose of QbD for HPLC methods, robustness and ruggedness should be verified early in the method development stage to ensure method performance over the lifetime of the product. Otherwise, if a non-robust or non-rugged method is adapted, significant time and resource may be required to redevelop, revalidate and retransfer analytical methods. According to literature survey, there are quite a few publications on HPLC method development strategy but the method development approaches for RP-HPLC specifically focused on pharmaceutical development in a QbD environment have not been widely discussed. Therefore, there is an unmet need to develop a systematic HPLC method development approach for pharmaceutical development using QbD principles to ensure the quality of the method throughout the product lifecycle. The aim of the analytical method is to separate and quantify the main compound while meeting the method performance criteria based on regulatory requirements, such as specificity, linearity, accuracy, precision, sensitivity, robustness, and ruggedness. The primary objective of this study was to implement Qbd approach to develop and validate an RP-HPLC method that could separate drug from its potential related substances and to establish an in depth understanding of the method

2.MATERIALS AND METHOD

Table No: 1 -Drug Name

Drug	**Bisoprolol Fumarate**
Source of drug	Unichem Lab, Goa

2.1 Chemicals:

Table No.2: Used other chemicals for the method development

Sr. no	Chemical use in method development	Mark
1	Water	Analytical grade
2	Methanol	HPLC Grade
3	Acetonitrile	HPLC Grade
4	Chloroform	HPLC Grade

2.2. Instruments:

Table No: 3- List of Instruments

Sr. No	Equipment Name	Source
1	UV visible spectroscopy	Shimadzu, Model: UV-1800
2	HPLC	Younglin (S.K) isocratic System
3	Digital weight balance	Shimadzu BL- 220 H
4	Hot air oven	Nisco company
5	Sonicator	The Ultrasonics PCi Analytics sonicator

Table.No. 4 Chromatographic Conditions

Sr. No.	Parameters	Chromatographic Conditions
1	Concentration	10 µg/ml
2	Column	C18 Cosmosil 4.6×150mm, 5µm
3	Mobile phase	Water; Methanol: Acetonitrile (50:30:20)
4	Wavelength	223 nm
5	Flow rate	1 ml/min
6	Run time	6 min
7	Injection time	20 µl
8	Temp.	36 ^{0}C
9	Detector	UV

3 EXPERIMENTAL METHOD

3.1. DRUG AUTHENTIFICATION [14]

3.1.1 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 100~102ºC and the melting point were noted.

3.1.2Solubility

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

Solution Preparation Method:

Preparation of Stock solution: Preparation of standard stock solution of Bisoprolol fumarate:

A stock standard solution was prepared by dissolving 0.010 mg of bisoprolol fumarate in a 10 ml of distilled water to obtain a concentration of 1000 µg/ml. appropriate concentration of 10 µ g/ml was prepared and scanned in the UV-visible over the range 400– 200 nm; Standard calibration solutions were prepared by dilution of the stock solutions using the diluent. These solutions were considered at six 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 Bisoprolol Fumarate was constructed by plotting the peak area against the drug concentration.

SELECTION OF DETECTION WAVELENGTH:

Fixing of wave length

After selecting the suitable solvent, the fixing of the λ max for the proposed method is very important. This can be done by scanning the drug sample (Bisoprolol fumarate) solution in distilled Water in the range of 400nm-200nm and the most repeated maximum absorbance with linearity and repeatability can be fixed as λ max for the drug. And in the proposed method for Bisoprolol fumarate drug shows maximum 223 nm. With more linearity, repeatability (ruggedness) and the λ_{max} was fixed as 223nm

Linearity and range:

For linearity study from the working standard at different concentration 2, 4, 6, 8, 10 and 12 µg/ml of drug solution were placed in 6 different 10ml volumetric flask volume was made up to the mark with bisoprolol fumarate. Absorbance was measured at 223nm. The obtained data of absorbance of standard stock solution presented in table no 10-calibration plot represented by Figure

Accuracy and recovery study:

This study was carried out using the stock solution (100µg/ml). Take three concentrations 8 µg/ml, 10µg/ml, and 12µg/ml and take six reading of these concentrations. Calculate the % Relative Standard Deviation (RSD) of the concentration. Results within the range of ensuring an accurate method as well as indicate non-interference with the excipients of formulation. The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100%, 120%. The recovery studies were carried out by adding known amount of Bisoprolol Fumarate solution of the drug to pre-analyzed tablet solutions. The resulting solutions were then reanalyzed by proposed methods

Intra-day precision (repeatability) and inter-day precision study (intermediate precision):

The standard stock solution of Bisoprolol was Prepared. Prepare the three concentration of (10µg/ml), by using mobile phase methanol. Take λ max at the intraday and inter day. Calculate the % RSD. Variation of results within the day (intra-day), Variation of result between days (inter day) were analyzed. Intraday precision was analyzing Bisoprolol Fumarate for three times in the same day at 223nm. Inter-day precision was determined by analyzing the drug different day for days at 223nm. Precision data for Bisoprolol Fumarate at 223nm.

Reproducibility:

Reproducibility is assessed by mean of an inter laboratory trial. The absorbance readings were measured at 223nm at different laboratory using another spectrophotometer and the value obtained were evaluated using t-test to verify their reproducibility data for Serotonin at 223nm is recorded.

Limit of Detection & Limit of Quantitation:

The limit of detection and quantification of drug are calculated with the standard deviation and slop.

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

Where,

σ = standard deviation

S = slope of calibration curve

Stability of Sample:

Samples prepared for repeatability study were preserved for 24hours at room temperature 28°C and analyzed on the following day to test for short-term stability. The Bisoprolol fumarate sample of 4µg/ml drug solution was prepared by suitable dilution with diluents and absorbance were taken at 223nm against the blank. The stability of sample was found to be more than 10 hrs.

Chromatographic Method Development by QbD Approach [15,16]

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.

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) is 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.

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) Stst-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.

Forced Degradation

a. Acid degradation:

The preparation of 0.01N hydrochloric acid (HCL) was done by diluting 0.085 ml of conc. HCL to 100 ml of distilled water as mentioned in IP. Bisoprolol Fumarate was accurately weighted and was transferred to a labeled round bottomed flask and add 10ml 0.01N hydrochloric acid (HCL). Reflux the sample for 2 hrs. Further make the dilutions to get final concentration 10µg/ml. Measure the absorbance in UV at 223 λ max.

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

b. Base degradation:

The preparation of 0.01N Sodium Hydroxide (NaOH) was done by dissolving 0.04 gm of sodium hydroxide pellets in 100 ml of distilled water as mentioned in IP. Bisoprolol Fumarate was accurately weighted and was transferred to a labeled round bottomed flask and add 10ml 0.01N Sodium Hydroxide (NaOH) Reflux the sample for 2 hrs. Further make the dilutions to get final concentration 10µg/ml. Measure the absorbance in UV at 223 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:

Weight accurately 10 mg drug and transferred in to10 ml water in round bottom flask. Reflux it for 2 hours. Reflux the sample for 2 hrs. Further make the dilutions to get final concentration 10µg/ml. Measure the absorbance in UV at 223 λ max.

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

d. Photo stability study:

Photo stability was performed by placing 10 mg of Bisoprolol Fumarate in daylight for 24 hours. The samples were diluted with mobile phase up to 100ml in a volumetric flask. Pipette out 1 ml sample diluted up to 10 ml by mobile phase. Measure the absorbance in UV at 223λ max.

e. Dry heat:

Standard quantity of Bisoprolol Fumarate was placed in an oven at 60C for 2 hours to observe degradation behavior of drug. To make stock solution (100 ppm) take 10 mg drug and dilute up to 100ml in a volumetric flask with mobile phase. Pipette out 1 ml and were diluted up to 10 ml by mobile phase. Measure the absorbance in UV at 223λ max.

f. Hydrogen peroxide degradation:

Weight accurately 10 mg drug and transferred in to10 ml (10%) H_2O_2 in round bottom flask. These samples were kept in a clean baker in a dark room for 2hours. Further make the dilutions to get final concentration 10µg/ml. Measure the absorbance in UV at 223 λ max.

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

4. RESULTS AND DISSCUSSION

Design of Experiment:

Table.No.5: 22 Factorial design with upper & 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	221nm	225 nm

The optimization phase was designed statistically using 22 factorial design in which two variables namely Flow rate, wavelength were 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 & lower limits factors Statistical sequence for optimization

Run No.	Replicates	Flow Rate	Wavelength	RT
1	5	1	223	4.3
2	8	1	225	4.33
3	4	0.8	223	5.3
4	2	1	221	4.4
5	3	1.2	221	5.6
6	6	1.2	223	4.9
7	7	0.8	225	4.94
8	1	0.8	221	4.9
9	9	1.2	225	4.5

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 stastical parameters including the coefficient of variation (CV), the coefficient of determination (R^2), adjusted coefficient of determination (adjusted R2) 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 3-5). 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 6) and graphical optimization technique by the overlay plot (Figure 6). To validate the chosen experimental design, the resultant experimental values of the responses were quantitatively compared with those of predicted values and the percentual 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 the 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)=+0.21*A+0.21009*B+0.011*AB

Table No.7 ANOVA for Response Surface Reduced Cubic Model Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.819E-003	6	4.698E-004	6.42	0.1409	significant
A-Flow Rate	*1.563E-005*	*1*	*1.563E-005*	*0.21*	*0.6893*	
B-Wavelength	*6.750E-006*	*1*	*6.750E-006*	*0.092*	*0.7900*	
AB	*5.131E-004*	*1*	*5.131E-004*	*7.01*	*0.1179*	
A^2	*1.830E-003*	*1*	*1.830E-003*	*25.01*	*0.0377*	
B^2	*1.231E-005*	*1*	*1.231E-005*	*0.17*	*0.7214*	
A^2B	*1.001E-004*	*1*	*1.001E-004*	*1.37*	*0.3627*	
Residual	1.463E-004	2	7.316E-005			
Cor Total	2.965E-003	8				

Std. Dev.	**8.553E-003**	**R-Squared**	**0.9507**
Mean	**0.21**	**Adj R-Squared**	**0.8026**
C.V. %	**4.07**	**Pred R-Squared**	**-0.4989**
PRESS	**4.444E-003**	**Adeq Precision**	**6.933**

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.23	1	6.375E-003	0.20	0.26	
A-Flow Rate	1.614E-003	1	3.492E-003	-0.013	0.017	1.00
B-Wavelength	1.837E-003	1	6.048E-003	-0.024	0.028	3.00
AB	0.011	1	4.277E-003	-7.075E-003	0.030	1.00
A^2	-0.030	1	6.048E-003	-0.056	-4.226E-003	1.00
B^2	2.481E-003	1	6.048E-003	-0.024	0.029	1.00
A^2B	8.663E-003	1	7.407E-003	-0.023	0.041	3.00

Externally Studentized Residual

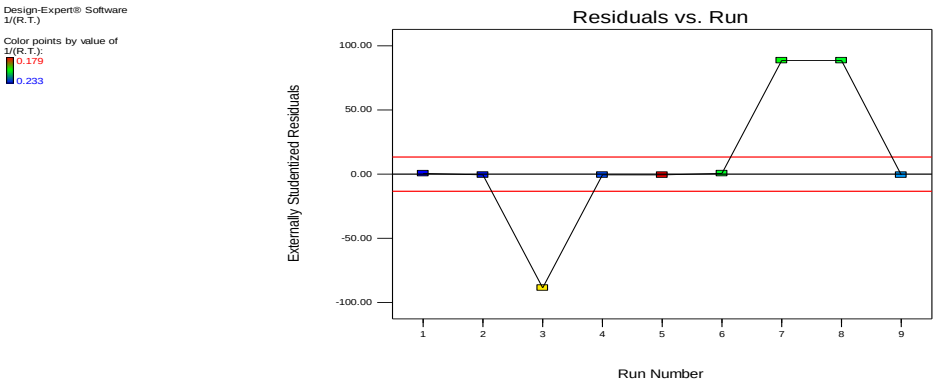

Figure No.1: Externally studentized residual graph of Residual Vs Run

Normal plot of Residuals

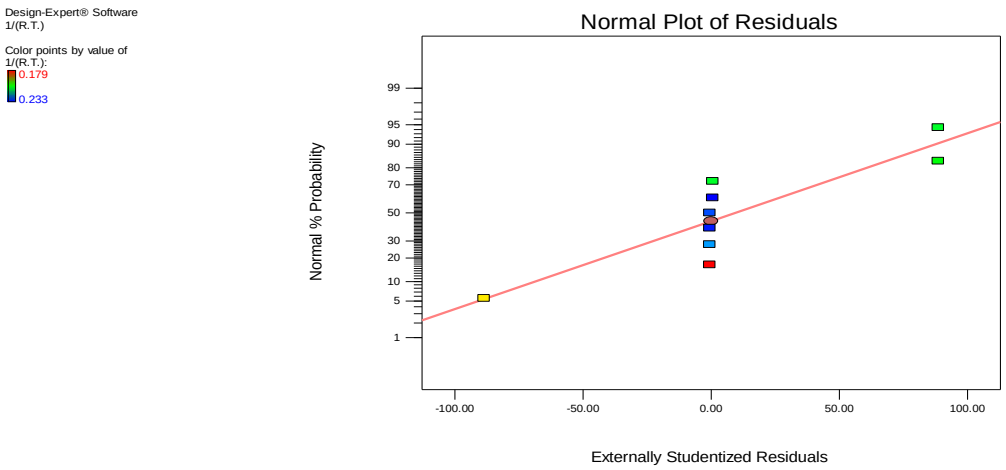

Figure No. 2: Externally studentized residual graph of Normal Vs Residuals

Predicted Vs Actual

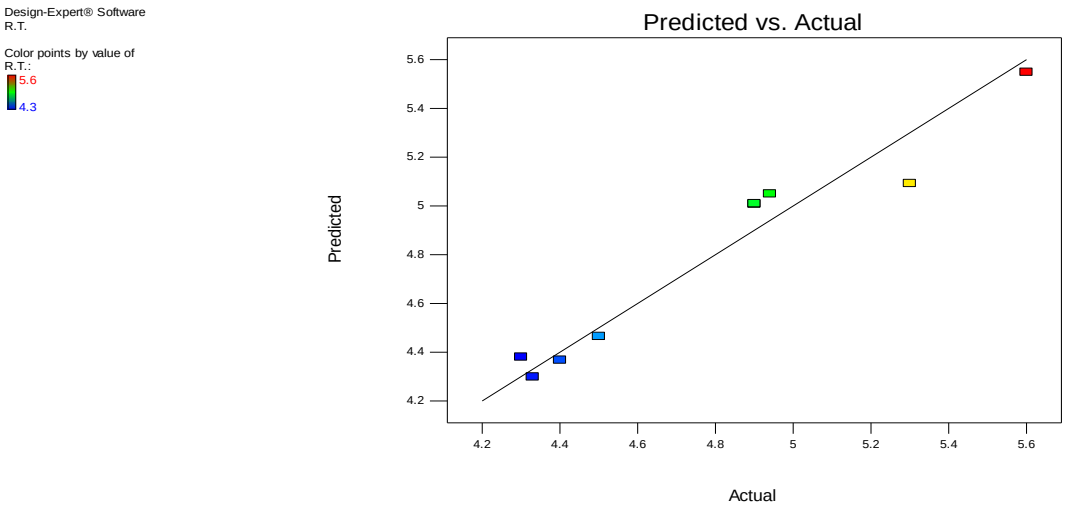

Figure No. 3: Externally studentized residual graph of Predicted Vs Actual

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

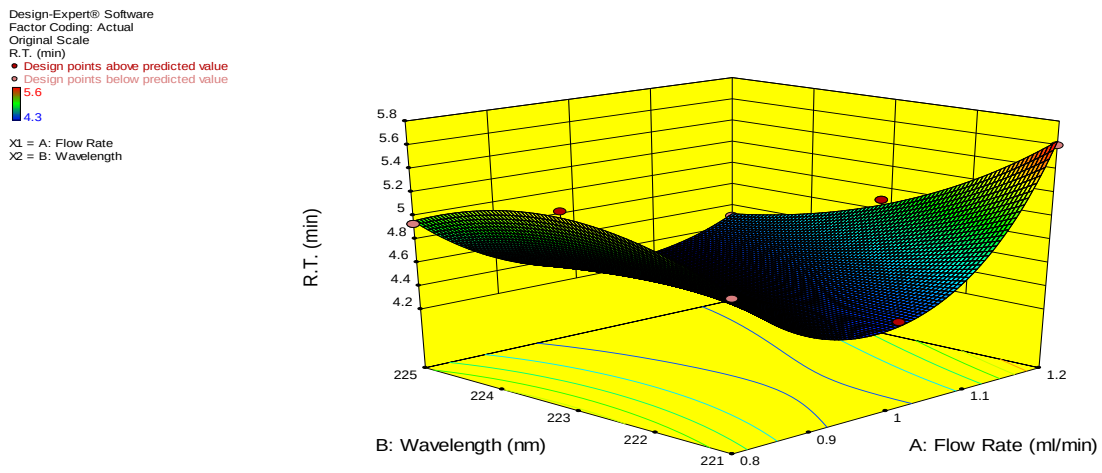

Figure 4: Response surface diagram showing combined effect of flow rate and wavelength on Rt of compounds at low level and high level (Counter Plot).

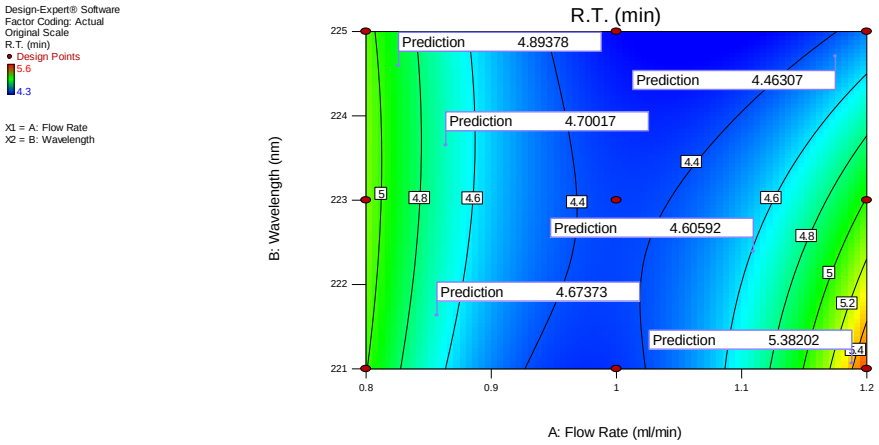

Figure No.5: Counter plot showing effect of flow rate and wavelength on Rt of compounds at low level
Desirability

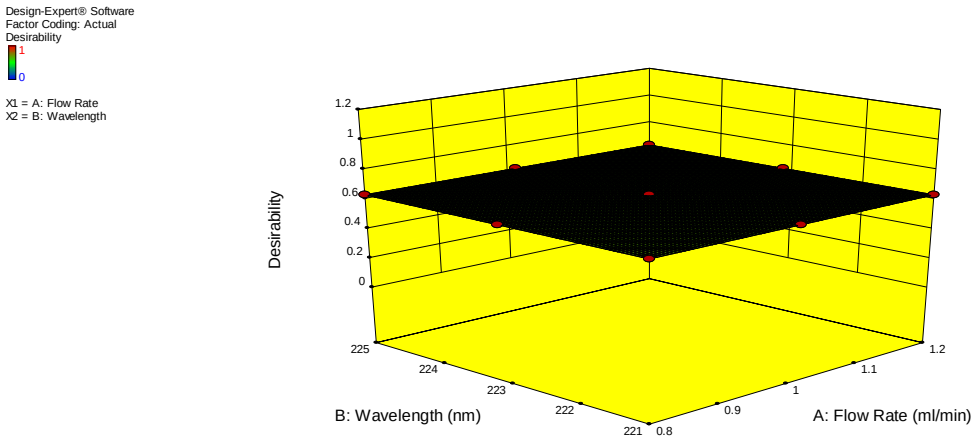

Figure No.6 Desirability

Table.No. 8 Chromatographic Conditions

Sr. No.	Parameters	Chromatographic Conditions
1	Concentration	10 µg/ml
2	Column	C18 Cosmosil 4.6×150mm, 5µm
3	Mobile phase	Water; Methanol: Acetonitrile (50:30:20)
4	Wavelength	223 nm
5	Flow rate	1 ml/min
6	Run time	6 min
7	Injection time	20 µl
8	Temp.	36 ^{0}C
9	Detector	UV

Standard chromatogram Of Bisoprolol Fumarate 10 µg/ml

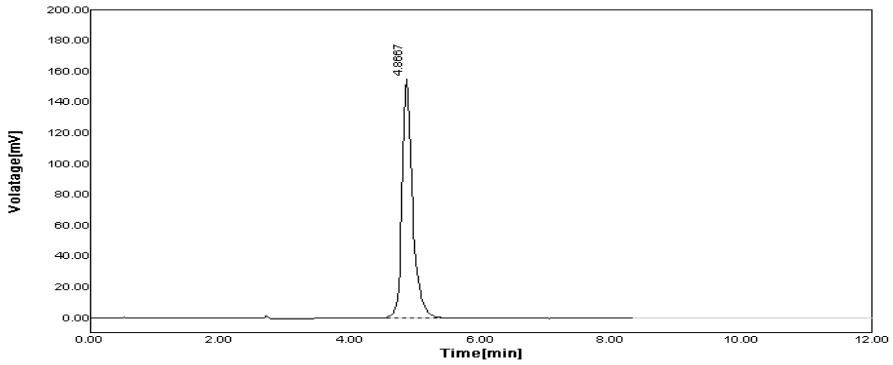

Figure No.9 Standard chromatogram

Linearity

Table No 9–linearity data

Sr. No	Concentration µg/ml	Area
1	2	552.46
2	4	817.35
3	6	1098.86
4	8	1375.83
5	10	1656.21
6	12	1878.12

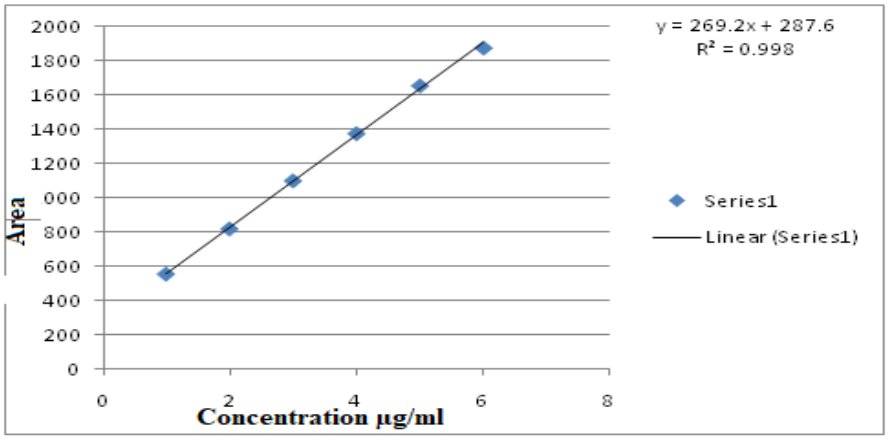

Figure No. 10 - Calibration curve

Table No 10 - Linearity parameter

Parameters	HPLC Method
Range	2-12 µg/ml
Slope	269.2
Intercept	287.6
Regretion Coefficient(R^2)	0.998

Accuracy

Table No 11–Accuracy Data

Accuracy	% Recovery	Average	Stastatical Analysis Mean	S.D	%R.S.D
80% 1	100.87	100.32	1260.26	18.52478	1.469911
80% 2	99.89				
80% 3	100.20				
100% 1	101.60				
100% 2	101.73	101.44	1385.752	0.161792	0.011675
100% 3	100.80				
100% 4	101.01				
100% 5	101.60				
100% 6	101.95				
120% 1	101.87	101.7	1510.813	9.730043	0.644001
120% 3	101.68				
120%3	101.55				

Precision

Table No 12-Intra-day precision Data

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	4.833	1379.955
2	10	4.826	1385.965
3	10	4.835	1399.97
4	10	4.830	1401.95
5	10	4.832	1550.96
6	10	4.837	1556.97
AVG.		4.8305	1445.962
S.D		0.00532	84.69071
%R.S.D		0.110129	0.581556

Table No 13 – Inter-day precision data

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	4.3667	1379.955
2	10	4.3661	1385.965
3	10	4.3659	1399.97
4	10	4.3662	1401.95
5	10	4.3630	1550.96
6	10	4.3601	1556.97
AVG.		4.36133	1649.781
S.D		0.010485	2.31507
%R.S.D		0.240412	0.14035

Robustness-

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

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	5.0099	1505.31
2	10	5.1011	1501.36
3	10	5.1001	1503.37
4	10	5.1011	1502.41
5	10	5.1001	11499.45
6	10	5.001	1489.50
AVG.		5.085383	1500.233
S.D		0.036983	5.611353
%R.S.D		0.727234	0.374032

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

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	4.1500	1536.57
2	10	4.1485	1540.61
3	10	4.1512	1539.7
4	10	4.1625	1585.41
5	10	4.1836	1591.49
6	10	4.1836	1595.78
AVG.		4.161383	1564.927
S. D		0.14267	28.6668
%R.S. D		0.342831	1.831822

Table No. 16-Change in the wavelength at 221nm

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	4.333	1166.4969
2	10	4.3395	1169.4978
3	10	4.3398	1145.4861
4	10	4.3285	1159.542
5	10	4.3211	1171.566
6	10	4.3451	1171.689
AVG.		4.3345	1165.213
S.D		0.008746	11.51783
%R.S.D		0.201767	0.988474

Table No. 17-Change in the wavelength at 225nm

Sr. No.	Concentration(µg/ml)	RT	Area
1	10	4.3500	1145.311
2	10	4.488	1153.325
3	10	4.450	1167.452
4	10	4.457	1139.446
5	10	4.468	1159.495
6	10	4.656	1157.471
AVG.		4.50316	1153.75
S.D		0.077192	10.10541
%R.S.D		1.714166	0.875875

Limit of Detection and Limit of Quantification

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where,

σ = standard deviation

S = slope of calibration curve

$$LOQ = \frac{10 \times \sigma}{S}$$

Where,

σ = standard deviation

S = slope of calibration curve

Table No.18 LOD & LOQ

LOD	0.0123(µg mL-1)
LOQ	10.6833(µg mL-1)

Analysis of Marketed Formulation

The peak at 4.333 was observed for Bisoprolol Fumarate in the chromatogram of the drug sample extracted from tablets. Experimental results of the amount of Bisoprolol Fumarate in tablets, expressed as percentage of label claim were in good agreement with the label claims thereby suggesting that here is no interference from any excipients, which are normally present in tablets. The drug content was found to be 100.32% and % RSD found to be is 0.057 for Bisoprolol Fumarate in tablet form.

Table No 19 – Analysis of Marketed Formulation

Sr. No.	Concentration (µg/ml	Area	% Accuracy
1	10	1658.3059	
2	10	1657.3161	
3	10	1661.3065	
4	10	1663.3062	**100.32%**
5	10	1664.3063	
6	10	1669.3064	
AVG.		1662.355	
S. D		4.47074	
%R.S.D		0.26894	

ASSAY-

The % purity of the bisoprolol Fumarate tablet was found to be 100.32% purity.

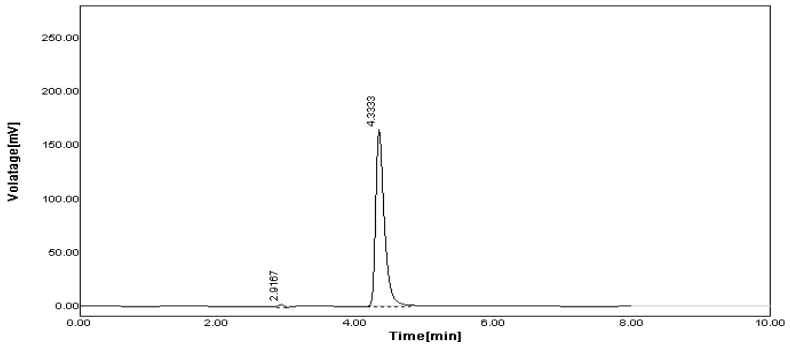

Figure No 11. Chromatogram for Assay.

Force Degradation

Degradation Chromatogram

0.1 N HCL

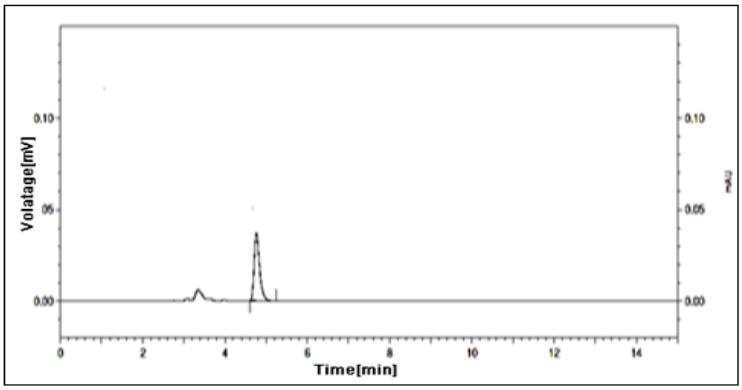

Figure No 12 - Chromatogram for 0.1 HCL

NaOH

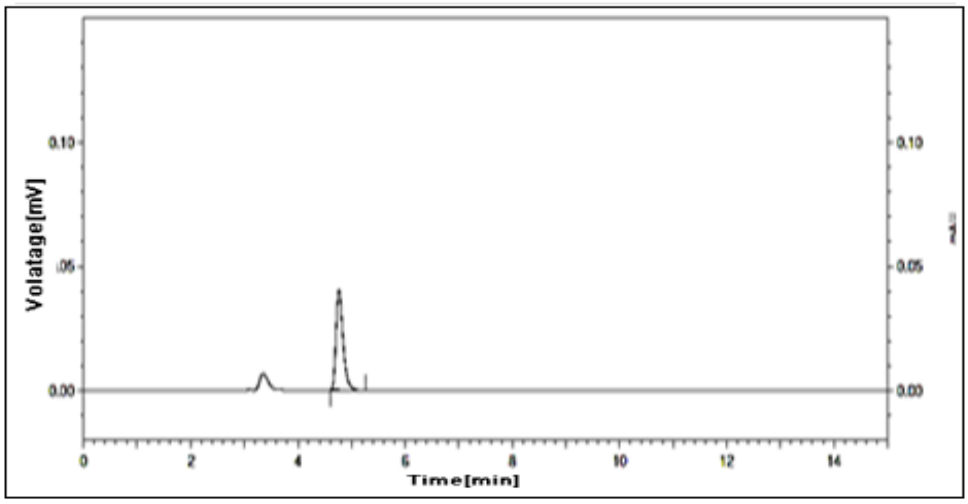

Figure No 13- Chromatogram for 0.1 N NaOH

OXIDATION

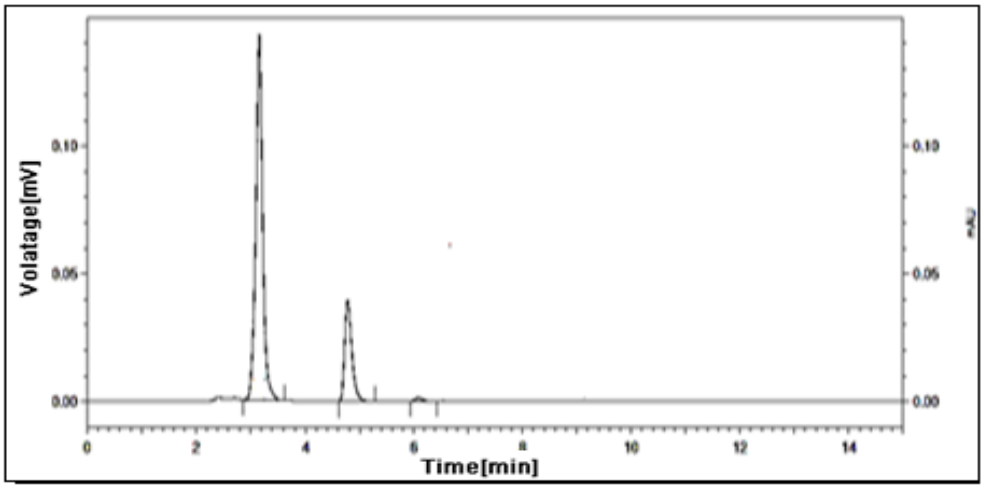

Figure No 14- Chromatogram for H_2O_2 OVEN AT 60 ^{0}C

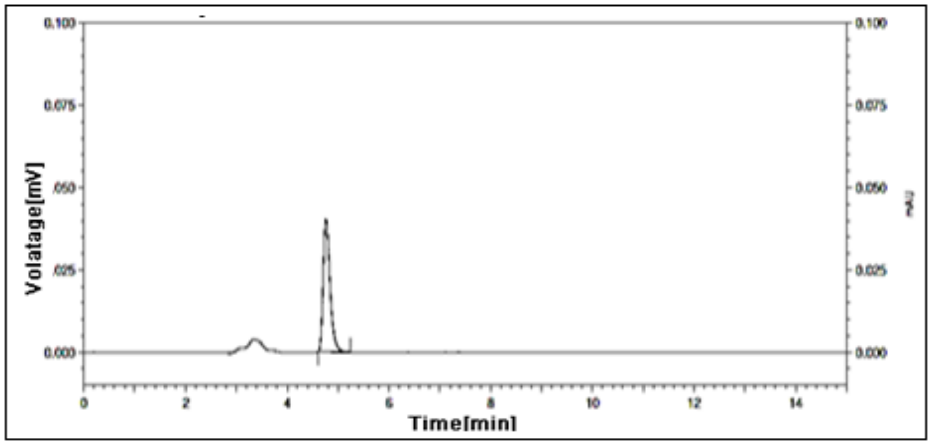

Figure No 15- Chromatogram for Oven at 60 ^{0}C

WATER

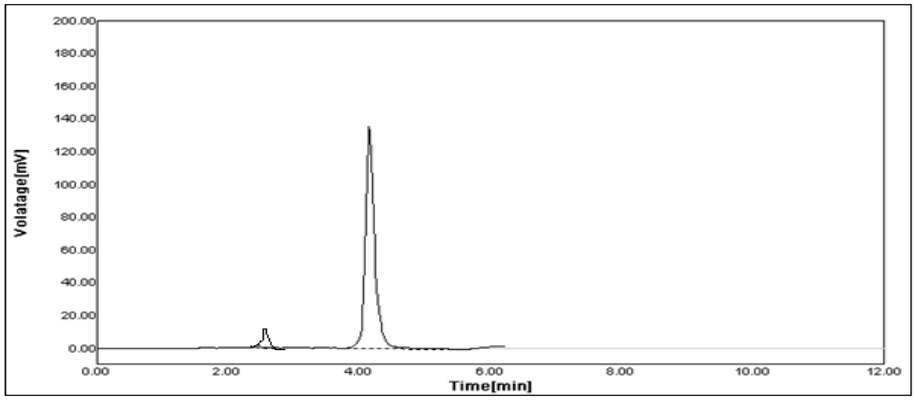

Figure No.16 Chromatogram for water

PHOTOLYTIC

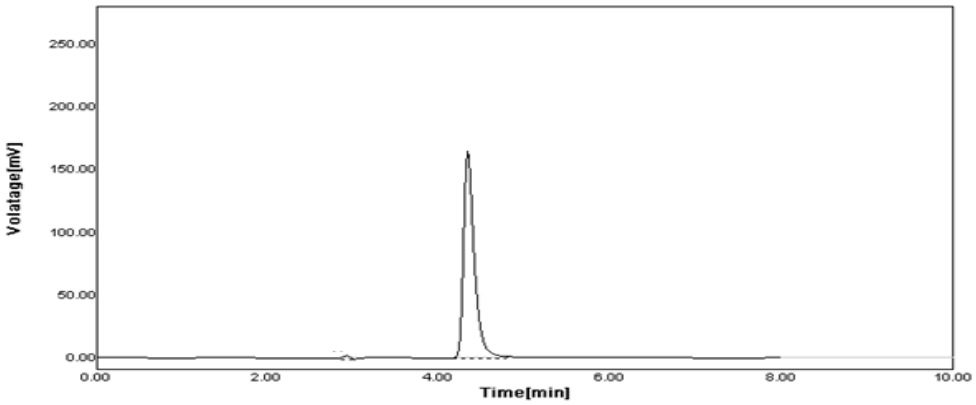

Figure No.17 Chromatogram for Photolytic

Force Degradation

Table No. 20: - Short term forced degradation data

Sr. No	Stress condition	% Degradation Observed	Remarks
1	0.1N NaOH	11.45%	Stable
2	0.1N HCL	10.47%	Stable
3	Oven	11.50%	Stable
4	Water	27.68%	Stable
5	Photolytic	9.30%	Stable
6	Oxidation	31.22%	Unstable

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 100.32 ± % 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

DISSCUSSION

Method2. A zero-order spectrophotometric method has been developed and validated for the determination of PFH in pharmaceutical formulation. QbD approach was carried out by varying 19 parameters and critical parameters were extracted by using principal component analysis and by observation.

A first order derivative spectrophotometric method has been developed and validated for the determination of PFH in pharmaceutical formulation. QbD approach was carried out by varying 27 parameters and extracted critical parameters by using principal component analysis and by observation.

Implementation of QbD approach was carried out by studying variable parameters in the analytical method development. Critical parameters were extracted by observation of results as well as performing principal component analysis. Also, each method was validated according to ICH Q2 (R1) guidelines. [10,12]

Table No. 48: Critical parameters extracted for method

Critical parameters extracted		By principal component analysis	
By observation Parameter	Extracted result	By observation Parameter	Extracted result
Solvent	Water	Wavelength	223 nm
Sample preparation	Tablet	Scan speed	Fast
		Slit width	1
		Sampling interval	0.2

Table 49: Statistical data of Validation

Parameter	Method
λ max	223 nm
Linearity range	2-12 µg/mL
Regression equation	Y=0.112x+0.004
Correlation coefficient	0.996
LOD	0.117 µg/mL
LOQ	0.35714µg/mL
Precision (%R.S. D)	
Intra-day	1.872
Inter-day	0.9495

A zero-order spectrophotometric method has been developed and validated for the determination of Bisoprolol Fumarate in pharmaceutical formulation. QbD approach was carried out by varying 19 parameters and critical parameters were extracted by using principal component analysis and by observation. [17,18] The extracted critical parameters are summarized in Table 48. Bisoprolol Fumarate followed linearity in the

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