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Quality by Design Based RP-HPLC for Simultaneous Estimation of Aspirin and Prasugrel HCL in Marketed Formulation

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Abstract:- Considering todays regulatory requirement for the development of an analytical method, a reversed phase high performance liquid chromatography method for simultaneous estimation of aspirin and prasugrel HCl in capsule dosage form has been optimized by analytical quality by design approach. Unlike the regular approach, this study began with the understanding of the objective profile of the quality product, the objective analytical profile and the risk assessment for the method variables that affect the response of the method. A liquid chromatography system equipped with a waters C_{18} column (150×3.9 mm, 5 µm), a isocratic delivery system (pump) and photodiode array detector were used to develop the method. The optimized method was achieved at 0.7 ml/min flow rate of using mobile phase of 1% Phosphoric acid buffer and Acetonitrile at 20:80(v/v), pH was adjusted to 3.5 with tri ethylene amine. To plan and analyses the experimental observations and obtain quadratic process model Design Expert software Version 11 was used. The process model was used for predicting solution for resolution. The optimized working condition was then validated according to ICH guidelines for linearity, LOD, LOQ, specificity.

Keywords:- *Quality by Design, Plackett-Burman, Box Behnken, HPLC, Aspirin, Prasugrel HCl.*

I. INTRODUCTION

Quality means fitness for the intended use.[1] Presently the pharmaceutical industry is adopting the QbD concept to improve the robustness of manufacturing processes and to facilitate continuous improvement strategies, to shape and improve product quality and manufacturing productivity. As per ICH Q8 (R2) quality by design is defined as *""A systematic approach to development that starts with predefined goals and focuses on understanding products and processes and controlling processes, based on sound and quality risk management."* [2] .

Aspirin is chemically 2-(acetyloxy) benzoic acid is cyclo oxygenase inhibitor and inhibits platelet aggregation while Prasugrel is [5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-6,7-dihydro-4H-thieno[3,2-c]pyridin-2-yl] acetate, is a member of the class of thienopyridines that inhibit the ADP receptor that induces platelet aggregation. Both agents act by binding irreversibly to P2Y12 receptors. Prasugrel acts more rapidly, consistently and to a greater extent in combination with aspirin.[5-6] Structure of Aspirin and Prasugrel are given as fig.1 and 2.

Literature survey revealed that few analytical methods such as RP-HPLC, LC-MS, UV and HPTLC have been reported for simultaneous estimation of Aspirin and Prasugrel HCl. QbD based RP- HPLC for simultaneous estimation of Aspirin and Prasugrel HCl in the marketed formulation has not been reported till date.[8-15] Hence the objective was to develop a simple, rapid, cost effective, sensitive, accurate, and precise RP-HPLC method for simultaneous estimation of Aspirin and Prasugrel HCl which was optimized by design of experiment software version 11. This work describes a simple, accurate, sensitive, accurate and validated method of simultaneous estimation of aspirin and prasugrel hydrochloride in the marketed formulation.

II. MATERIALS AND METHODS

- *Chemicals and Reagents:*

Bulk drugs Prasugrel HCl was obtained as a gift sample from MYLAN and Aspirin from ACROS ORGANICS. Acetonitrile, o-Phosphoric acid, Tri-ethylene amine were obtain from Merck and Milli-Q water of HPLC grade was used for the analysis.

- *Stock and Standard solution:*

75mg of Aspirin and 10 mg of Prasugrel HCl were weighed accurately and transferred to 100ml volumetric flask to it 50 ml of 1% o- phosphoric acid buffer and acetonitrile (50:50 v/v) was added, sonication for 10 minutes then the volume was made to the mark. This gives the standard stock solution having concentration of 750µg/ml of Aspirin and 100 µg/ml of Prasugrel HCl.

- *Preparation of Calibration Curve:*

Stock solution was prepared by dissolving 10mg of Aspirin and Prasugrel HCl in 100ml volumetric flask, 50

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ml of mobile phase was added, sonicated for 10 minutes then the volume was made to the mark to get the concentration of 100 µg/ml of each. Subsequent dilutions were made from the above solution to get concentration ranging from 60 µg/ml to 90 µg/ml of aspirin and 80 µg/ml to 120µg/ml of Prasugrel HCl.

➢ *Chromatographic condition:*

HPLC analysis was performed on SHIMATZU 10 AD HPLC with SPD 10 A UV-Vis detector. The Software equipped was Class VP . The chromatographic Column, waters C-18 (150 x 3.9mm, 5 micrometer) was used as a stationary phase and the mobile phase was 1% o-Phosphoric acid buffer and Acetonitrile pH adjusted to 3.5 with tri-ethylene amine in the ratio of 20:80 . The pump flow rate was 0.5ml/min and injection volume was set at 20µl. The eluent was detected at 254nm with the run time of 10 minutes.

➢ *Preparation of sample solution:*

20 capsules were weighed to determine the average weight and tablet within were crushed to form a fine powder. 0.4g of crushed powder was weighed accurately and transferred in to a 100 ml of volumetric flask, 50ml of mobile phase was added, sonicated for 10min and volume was made to mark with mobile phase. This solution was centrifuged for 10 minutes 1000rpm. Solution was filtered through a 0.45µ Millipore filter and marked as test solution.

➢ *Method design:*

1) Screening Method:- Screening was done using Plackett-Burman Design using Design Expert Software 11.

The Following 5 factors were selected.

- Acetonitrile Concentration
- Detection Wavelength
- Column Oven temperature
- Flow Rate
- Injection Volume

Total 12 runs were obtained, the response for design was resolution the peaks of the drugs. Results were put in design to further optimize the method. Table. 1 and 2.

2) Optimization:- response surface methodology was used, applying a 3 level Box Behnken design with 3 center points (Table 3). Factor selected were injection volume, flow rate, column temperature. Evaluation of main factor, their interaction and quadric effect on peak resolution were done. Acetonitrile concentration 80% and wavelength were kept constant as their effect on resolution was less significant. Experiments were conducted by injections of standard drug solutions and the average of resolution was analyzed by Design Expert 11 software**.** (Table 4).

Multivariate regression analysis application resulted in a fitted full quadrate model for the average responses of peaks USP resolution given by the equation 1.

$$Y= \beta 0 +\beta 1 X 1 +\beta 2 X 2 + \beta 3 X 3 + \beta 11 X 1 2 + \beta 22 X 2 2 + \beta 33 X 3 2 + \beta 12 X 1 X 2 + \beta 13 X 1 X 3 + \beta 23 X 2 X 3 \quad (1)$$

Where Y is the response, β 0 is the arithmetic mean response. B 1 β 2 and β 3 are regression coefficients of factor X 1, X 2 and X 3 respectively. B 11, β 22 β 33 are square coefficients β 12, β 13 and β 23 are interaction coefficients.

➢ *Assay of Marketed Formulation:*

Prepared sample solution was injected under optimized chromatographic conditions and chromatograms were recorded. The amount of Aspirin and Prasugrel HCl is calculated from the calibration curve.

➢ *Validation of Method:*

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) [12] guidelines for linearity, LOD and LOQ, specificity.

- *Linearity:*

As per ICH guidelines the linearity of analytical procedure is its ability (within in a given range) to obtain test results directly proportional to the concentration (amount) of analyte in sample. 10mg of Aspirin and Prasugrel HCl were dissolved in 100ml of mobile phase, sonicated for 10 minutes followed by filtration through 0.45µ Millipore filter. Standard calibration curves were prepared with five different concentrations, over the range of 60, 67, 75, 82, 90µg/ml for Aspirin and 80, 90, 100, 110, 120µg/ml for Prasugrel HCl. Linear concentration curves of peak area versus drug concentration were plotted using linear least squares regression and evaluated for linearity. [Table: 10,11]

- *LOD and LOQ:*

Limit of detection (LOD) and Limit of Quantification (LOQ) were experimentally determined and calculated mathematically using the following formulae:

$$LOD= \frac{3.3 \text{ standard deviation of y intercept}}{\text{Slope of calibration curve.}}$$

$$LOQ= \frac{10 \text{ standard deviation of y intercept}}{\text{Slope of calibration curve.}}$$

LOD and LOQ were experimentally verified by diluting known concentration of a sample solution. [Table 12]

- *Specificity:*

The specificity of the method was determined by comparing the bulk drug chromatogram with that of the marketed formulation at an equivalent concentration using the optimized method. The drug spots were confirmed by comparing the value of Rf with the spectra of the spot in the sample with the standard. The peak purity of the sample was analyzed by comparing the spectra at the

beginning of the peak, the maximum apex and the maximum final position of the point. [Fig.10]

III. RESULTS AND DISCUSSION

A. Preliminary Study:

Optimization of mobile phase were done on reverse phase C_{18} column (3.9 x 150 mm, 5µm particle size) starting with 0.01M Potassium di Hydrogen Phosphate buffer: Acetonitrile, 0.01 M Ammonium Di Hydrogen Phosphate buffer pH7.9: Acetonitrile , 1% o- Phosphoric Acid Buffer pH 2.5: Acetonitrile.). After several trial it was found that 1% o- Phosphoric Acid Buffer pH 3.5: Acetonitrile (20:80) gave better results as compared to other mobile phases. Peaks were obtained with the resolution of 3.69, flow rate of 0.5 ml/min, column temperature 25˚C at 254 wavelength. Further screening was done using Plackett- Burman and optimization was done by carrying out runs by box Behnken design.

B. Method Design:

- *Plackett Burman*:- Five factors were selected and screened with six dummy factors. The runs obtained were 12 with cross combination, the response of these runs was resolution which was obtained through these 12 runs and the method was optimized (Table.5). Three factors affecting the resolution were obtained from Pareto chart. (Fig. 3) Further optimization was done using Box Behnken.

- *Box Behnken design:*

i) Analysis of variance as dependent variable for Resolution response

Application of multivariate regression analysis gave fitted full quadratic model for the USP Resolution of peaks. Factors considered here are flow rate, injection volume, column temperature. Regression coefficient and p-values obtained from the report generated by the software are given in (Table. 6)

An analysis of variance (ANOVA) was performed to study the importance of the factors and terms of interaction on the response i.e. resolution between the peak, p-value simply provide the cut-off beyond which we assert that the findings are ‘statistically significant’ by convention, it is $p<0.05$.

A value of Probe > F was found to be less than 0.05, therefore, the model was found to be significant for predicting the response. The whole model has been well adjusted for optimization. Also a lack fit was significant. Factors were found to be significant i.e. flow rate($p=0.0052$) and column temperature($p=0.008$), interaction, flow rate x column temperature ($p=0.0061$), injection volume x injection volume ($p=0.0014$), flow rate x flow rate ($p=<0.0072$), Column temperature x column temperature ($p<0.0087$).While the interactions of injection volume x flow rate ($p=1.0000$), injection volume x column temperature ($p=0.8161$), were found to be insignificant.

ii) Model Assessment for Resolution Response as Dependent Variable:-

3 of the factor were found to affect the response i.e. resolution from their respective coefficients. Injection volume, interaction of injection volume x flow rate, injection, volume x column temperature, flow rate x column temperature are showing inverse relationship with resolution.

iii) Response surface plot

The response surface and the contour plot were studied to visualize the effect of the factor and its interaction in order to develop a design space for a robust method 3D graph are given below in fig no. 4, 5, 6.

From the graph you can find some facts about the effect of the factors and their interaction in the response. The curvature in the contour graph shows a non-linear relationship between the factors. From (Fig.4) showing effect of injection volume and flow rate. (where wavelength is constant at 254nm), it can be observed that resolution was as per specification when flow rate of 0.3-0.7 ml/min and injection volume is between 8-12µl. If injection volume and flow rate is increased then the resolution gets affected.

Keeping the flow rate is constant at 0.5ml/min, effects of injection volume and column temperature were studied.(Fig. 5) It was found that at injection volume between 8-12µl and column temperature should be between 23-27˚C, resolution was as per specification otherwise gets decreased.

When injection volume was kept constant at 10µl and flow rate and column temperature were studied. Flow rate should be in between 0.3-0.8ml/min and the column temperature between 23-27˚C. (Fig. 6). From these three of diagrams it can be concluded flow rate should be between 0.3-0.8ml/min, injection volume 8-12µl has more effect on.

C. Optimized Condition

To obtain optimum set of conditions to achieve desirable goal composite desirability parameters were applied. Response set to resolution between Aspirin and Prasugrel HCl was 6.40. Optimum conditions having desirability was chosen from the obtained runs i.e. Flow rate 0.7 ml/ min, Injection volume 10 µl, Column temperature 23 ˚C. Set of conditions were analyzed to compare predicted response with the actual response. (Table. 7)

D. Application of optimized method for Assay of marketed formulation:

(Table 8 and Fig. 7)

E. Solutions for the optimized batch

(Table 9)

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F. Validation of optimized method:

Method validation was done according to the ICH guideline Q2 [12]. The results were within the specified limit and the optimized method was found to be accurate and precise.

➢ *Linearity:*

1) Aspirin

The linearity of aspirin was obtained over the concentration ranging from 60-*90 µg/*ml. The regression equation was found to be y = 7775.3x - 45218.The regression coefficient was found to be R^2 = 0.994 (Table 10, fig. 8)

2) Prasugrel HCl:

The linearity of Prasugrel HCl was obtained over the concentration ranging from 80-120 µg/ml. The regression equation was found to be y = 2914.5x - 20256. The regression coefficient was found to be R^2 = 0.9942. (Table 11, fig. 9)

- *LOD and LOQ:*

The LOD and LOQ parameters were evaluated using the slope of the line and the standard deviation of the calibration studies. (Table 12)

- *Specificity:*

The peak purity of aspirin and Prasugrel HCl were evaluated by comparing the spectra of the standard and the sample at the beginning of the peak, the maximum peak and the maximum final positions of the point, their spectra were superimposed to evaluate the spectral correspondence. Any degraded product that may be present was observed in chromatogram studies and easily distinguished from the pure pharmacological substance. (Table 12, fig. 10)

IV. CONCLUSION

- Approach to the Quality by Design approach has been successfully applied for Simultaneous Estimation of aspirin and prasugrel HCl in capsule dosage form by the RP-HPLC method. All key aspects of QbD were attempted to implement in that study.
- In RP-HPLC method development, optimization was carried out by applying QbD and the parameters optimized were flow rate of 0.7 ml/min, column temperature 23˚C, mobile phase buffer (0.1% o-phosphoric acid pH 3.5): Acetonitrile (20:80 v/v) and wavelength at 254 nm , injection volume 10 µl which give sharp peaks with the resolution of 6.40.
- The method developed for the Simultaneous Estimation of Aspirin and Prasugrel was validated according to the ICH Q2 (R1) guidelines using various parameters such as, linearity, precision, specificity, LOD, LOQ, accuracy.
- The method developed based on QbD can be applied for the routine quality control of the combined marketed formulation.

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TABLES AND FIGURES

Sr. No.	Chromatographic Condition	Levels used		
		Low	Center	High
1	Acetonitrile Concentration (%)	75	80	85
2	Detection Wavelength (nm)	249	254	259
3	Column Oven temperature (°C)	20	25	30
4	Flow Rate (ml/min.)	0.1	0.5	0.9
5	Injection Volume (µL)	5	10	15

TABLE 1: CHROMATOGRAPHIC FACTORS AND RESPONSE VARIABLES FOR PLACKETT-BURMAN EXPERIMENTAL DESIGN.
%-percent, nm-nanometer, °C-degree Celsius, ml/min- milli liter per minute, µL- micro Liter.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Run	A:Acetonitrile	B:Wavelength	C:Injection Volume	D:Flow Rate	E:Column Temperature
	%	Nm	µl	ml/min	°C
1	85	259	5	0.1	20
2	85	249	15	0.9	30
3	75	259	15	0.9	20
4	75	259	15	0.1	30
5	85	249	15	0.9	20
6	75	249	5	0.1	20
7	75	259	5	0.9	30
8	85	249	5	0.1	30
9	75	249	5	0.9	20
10	85	259	15	0.1	20
11	75	249	15	0.1	30
12	85	259	5	0.9	30

TABLE 2: PLACKETT BURMAN METHOD USED FOR ASPIRIN AND PRASUGREL HCL SCREENING
%-percent, nm-nanometer, °C-degree Celsius, ml/min- milli liter per minute, µL- micro Liter.

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Chromatographic Condition	Levels		
	Low	Center	High
Injection Volume (µl)	8	10	12
Flow Rate (ml/min)	0.3	0.5	0.7
Column Temperature (˚C)	23	25	27

TABLE 3CHROMATOGRAPHIC FACTORS AND RESPONSE VARIABLES FOR BOX BEHNKEN DESIGN
µL- micro Liter, ml/min- milli liter per minute, °C-degree Celsius.

Run	Coded (X_1, X_2, X_3)	Injection Volume(µL)	Flow Rate (ml/min)	Column Temperature (°C)
1	+-0	12	0.3	25
2	++0	12	0.7	25
3	--0	8	0.3	25
4	0+0	10	0.5	25
5	+0-	12	0.5	23
6	000	10	0.5	25
7	000	10	0.5	25
8	0+-	10	0.7	23
9	-0+	8	0.5	27
10	000	10	0.5	25
11	-+0	8	0.7	25
12	0--	10	0.3	23
13	0++	10	0.7	27
14	+0+	12	0.5	27
15	-0-	8	0.5	23
16	0-+	10	0.3	27
17	000	10	0.5	25

TABLE 4: BOX BEHNKEN DESIGN USED FOR ASPIRIN AND PRASUGREL HCL OPTIMIZATION
'+'indicates high value, '-'indicates low value and '0' is the center, µL- micro Liter, , ml/min- milli liter per minute, °C-degree Celsius.

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	6.21	2.07	18.08	0.0006	Significant
C-Injection Volume	4.78	4.78	41.69	0.0002	
D-Flow Rate	0.806	0.806	7.04	0.0291	
E-Column Temperature	0.6302	0.6302	5.5	0.047	
Residual	0.9163	0.1145			
Cor Total	7.13				

TABLE 5: REGRESSION COEFFICIENTS AND ASSOCIATED PROBABILITY VALUES (P-VALUES) FOR USP RESOLUTION OF ASPIRIN AND PRASUGREL HCl (Plackett Burman)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	16.13	1.79	11.58	0.002	significant
A-INJECTION VOLUME	0.0351	0.0351	0.2268	0.6484	
B-FLOW RATE	2.48	2.48	15.99	0.0052	
C-COLUMN TEMP	2.08	2.08	13.44	0.008	
AB	0	0	0	1	
AC	0.009	0.009	0.0583	0.8161	
BC	2.33	2.33	15.02	0.0061	
A^2	4.08	4.08	26.34	0.0014	
B^2	2.18	2.18	14.06	0.0072	
C^2	2.01	2.01	13.01	0.0087	
Residual	1.08	0.1548			
Lack of Fit	1.05	0.3493	39.16	0.002	significant
Pure Error	0.0357	0.0089			
Cor Total	17.21				

TABLE 6: REGRESSION COEFFICIENTS AND ASSOCIATED PROBABILITY VALUES (P-VALUES) FOR USP RESOLUTION OF ASPIRIN AND PRASUGREL. HCl (Box Behnken)
AB injection volume x Flow rate, AC-injection volume x Column temperature, BC- flow rate x column temperature.,

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Flow rate (ml/ min)	Injection volume (µl)	Column temperature (˚C)
0.7 (ml/ min)	10 (µl)	23 (˚C)

TABLE 7: HPLC OPTIMIZED BATCH OF ASPIRIN AND PRASUGREL HCL.
µL- micro liter, ml/min- milli liter per minute, °C-degree Celsius.

Sr. No.	Drugs	Label claim (mg)	Concentration taken (µg/ml)	Area	Amount found (µg/ml)	% Assay
1	Aspirin	75 mg	750 µg/ml	5476180	745.08 µg/ml	99.34%
2	Prasugrel HCl	10mg	100 µg/ml	277514	102.97 µg/ml	102.9%

TABLE 8: ASSAY RESULTS OF MARKETED FORMULATION OF ASPIRIN AND PRASUGREL HCL
µg/ml- microgram per milli liter

Number	INJECTION VOLUME	FLOW RATE	COLUMN TEMP	RESOLUTION	Desirability	
1	*8.319*	*0.353*	*26.312*	4.26	1	
2	*12*	*0.7*	*25*	4.775	1	
3	*10*	*0.3*	*27*	4.274	1	
4	*12*	*0.5*	*23*	4.709	1	
5	*8*	*0.7*	*25*	4.907	1	
6	*8*	*0.3*	*25*	3.795	1	
7	***10***	***0.7***	***23***	**6.406**	**1**	**Selected**
8	*10*	*0.7*	*27*	3.861	1	
9	*10*	*0.3*	*23*	3.769	1	
10	*8*	*0.5*	*23*	4.936	1	
11	*8*	*0.5*	*27*	3.821	1	
12	*12*	*0.3*	*25*	3.663	1	
13	*12*	*0.5*	*27*	3.784	1	
14	*11.259*	*0.514*	*24.078*	5.69	1	
15	*10.056*	*0.613*	*26.615*	4.862	1	
16	*11.257*	*0.326*	*24.765*	4.496	1	
17	*8.644*	*0.629*	*24.753*	5.757	1	
18	*8.948*	*0.542*	*23.688*	5.995	1	
19	*9.695*	*0.426*	*26.309*	5.221	1	
20	*8.41*	*0.35*	*24.073*	4.439	1	
21	*8.451*	*0.336*	*24.194*	4.363	1	
22	*8.64*	*0.524*	*26.403*	4.85	1	
23	*11.454*	*0.356*	*25.186*	4.645	1	
24	*11.24*	*0.335*	*26.66*	4.269	1	
25	*11.258*	*0.695*	*25.481*	5.083	1	

TABLE 9: SOLUTIONS FOR THE OPTIMIZED BATCH.

Concentration of stand. In µg/ml	Area
00	00
60.0	429802.0
67.0	468719.0
75.0	534122.0
82.0	587730.0
90.0	661481.0

TABLE 10: HPLC LINEARITY DATA OF ASPIRIN
µg/ml- microgram per milli liter

Concentration of stand. In ***µg*/ml**	Area
80	216184.0
90	239833.0
100	269904.0
110	296461.0
120	333596.0

TABLE 11: HPLC LINEARITY DATA OF PRASUGREL HCL
µg/ml- microgram per milli Liter

Sr. No.	Parameter	Data for Aspirin	Data for Prasugrel HCl
1.	Linearity	60-90µg/ml	80-120µg/ml
2.	Regressions equation	y = 7775.3x - 45218	y = 2914.5x - 20256
3.	Correlation coefficient (r^2)	0.994	0.994
4.	LOD(µg/mL)	16.82324	24.16456
5.	LOQ(µg/mL)	50.9795	73.22594
6.	Specificity	Specific	

TABLE 12: VALIDATION PARAMETERS OF HPLC METHOD..
µg/ml- microgram per milli liter, LOD-limit of detection, LOQ- Limit of Quantification.

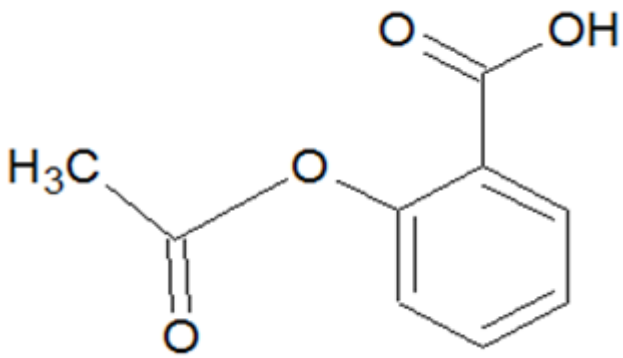

Fig. 1: Structure of Aspirin

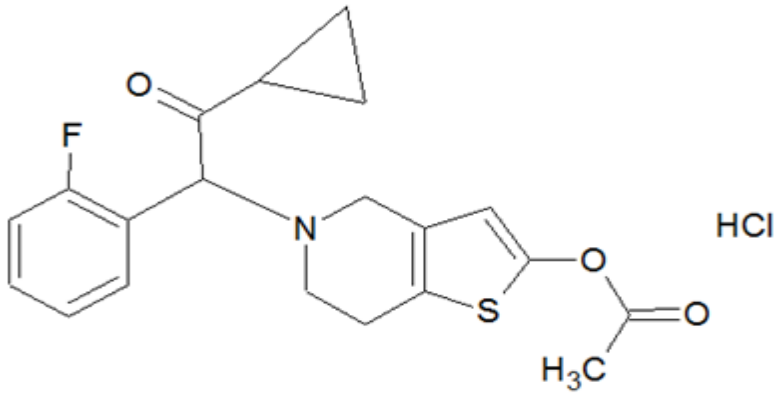

Fig. 1: Structure of Prasugrel HCl

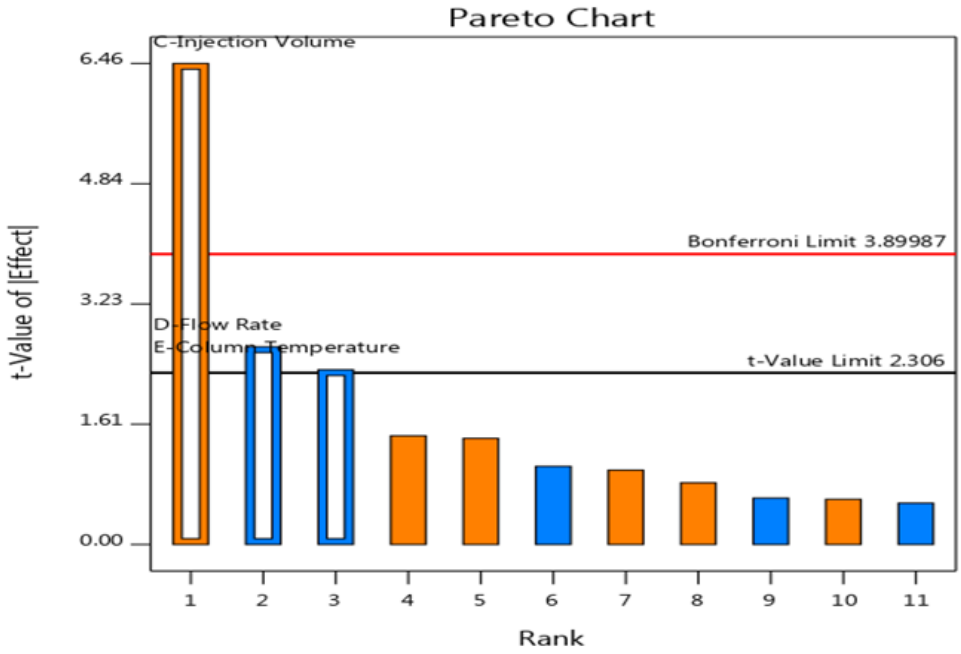

Fig 3: Pareto Chart ranking.

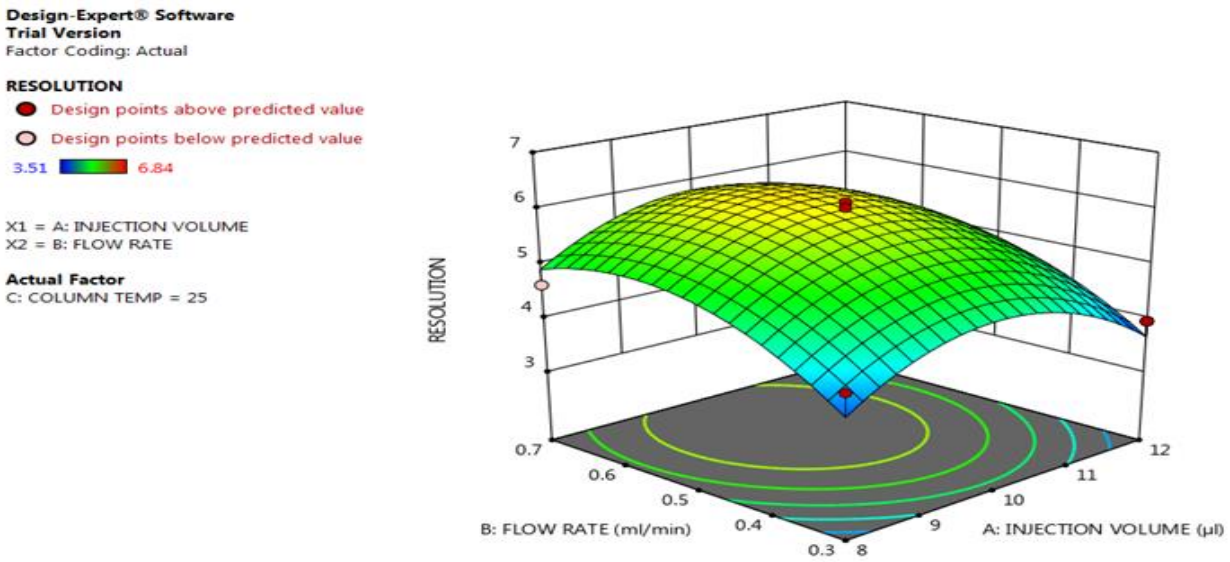

Fig. 4: Response plot (3D) Showing Effects of injection volume and flow rate on USP Resolution factor of Aspirin and Prasugrel HCl.

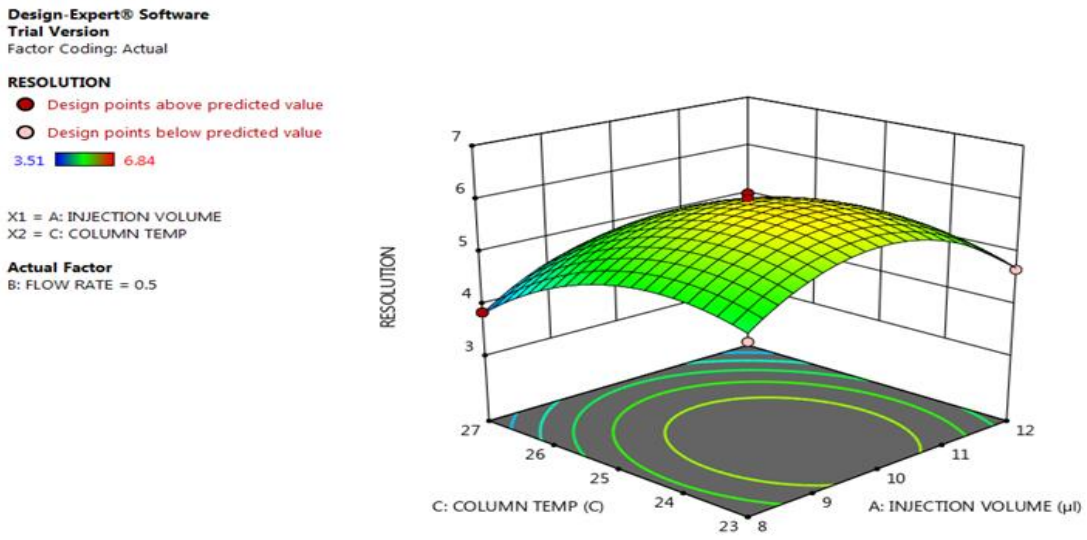

Fig. 5: Response plot (3D) Showing Effects of injection volume and Column temperature on USP Resolution factor of Aspirin and Prasugrel HCl.

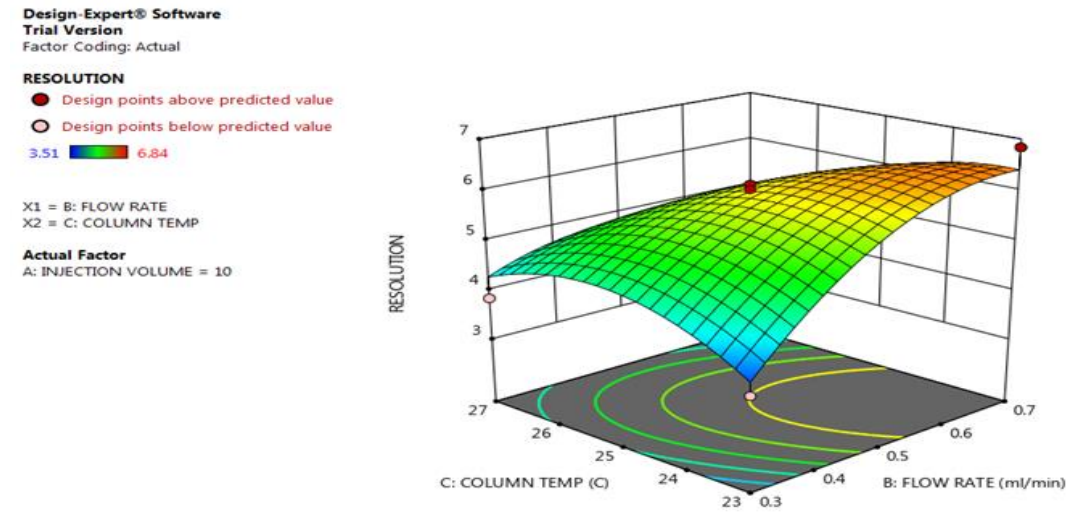

Fig. 6: Response plot (3D) Showing Effects of Column temperature and flow rate on USP Resolution factor of Aspirin and Prasugrel HCl.

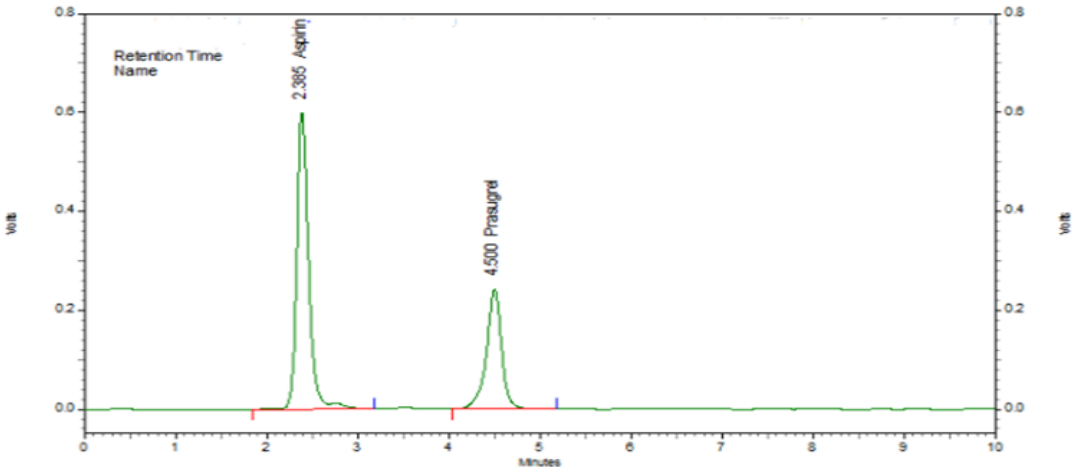

Fig.7: Chromatogram of optimized condition of marketed formulation.
Rt:- retention time.
Aspirin (peak 1) with Rt 2.385 minutes, Prasugrel HCl (Peak 2) with Rt 4.500 minutes.

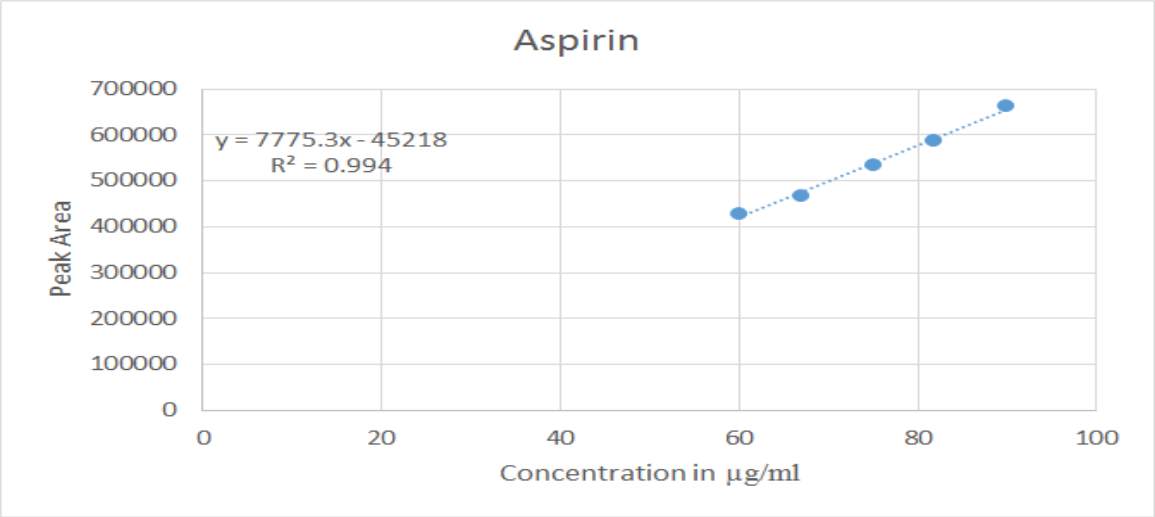

Fig. 8: Linearity plot of aspirin.

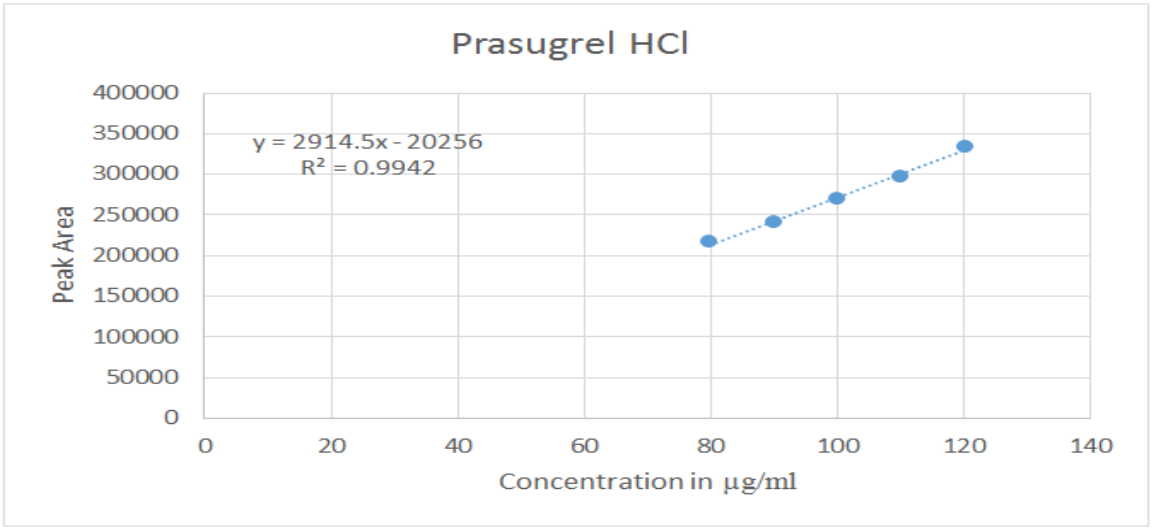

Fig. 9: Linearity plot of Prasugrel HCl.

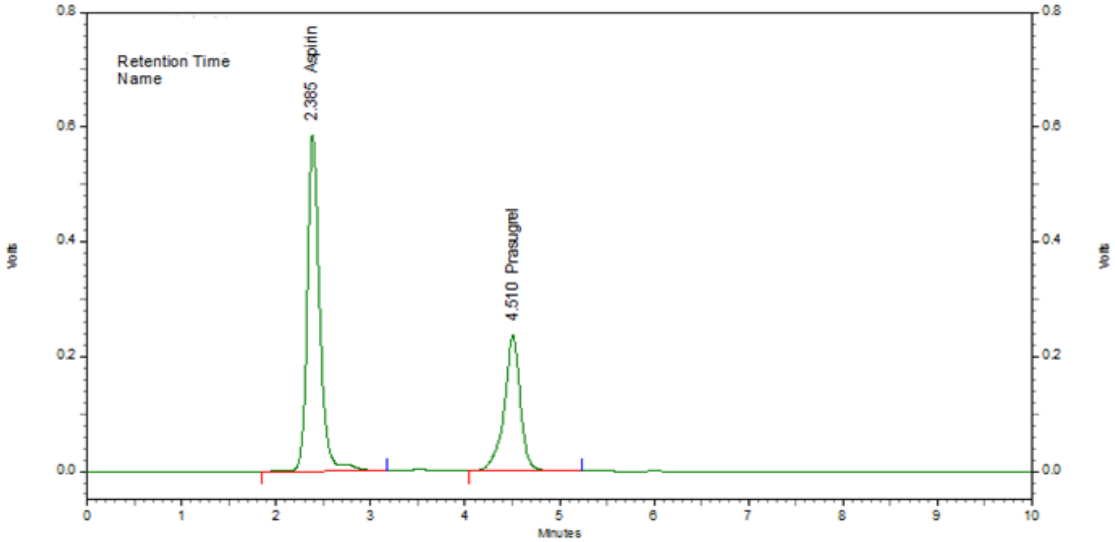

Fig. 10: Chromatogram of specificity of Aspirin and Prasugrel HCl in marketed formulation.
Rt- retention time,
Aspirin (peak 1) with Rt 2.385 minutes, Prasugrel HCl (Peak 2) with Rt 4.501 minutes.