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CHEMOMETRICS ASSISTED METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING LIQUID CHROMATOGRAPHY METHOD FOR THE ESTIMATION OF BUDESONIDE IN DOSAGE FORMULATION

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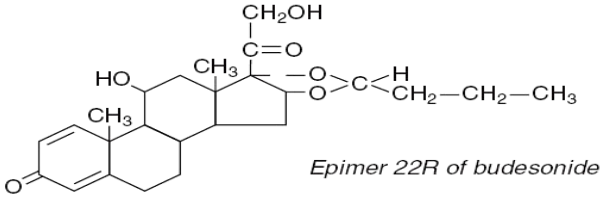
ABSTRACT
Development and validation of a novel and sensitive high performance liquid chromatography method for the estimation of Budesonide (BDS) in tablet dosage form was established by using quality by design approach (QbD). The development activity is focused by the LC technique by UV detection using base deactivated columns.Validation procedure is intended and accomplished as per International Conference on Harmonization (ICH) guidelines. The method uses a Thermo Hypersil BDS C18, 150 x 4.6 mm, 3.0 µm column in isocratic elution with a mobile phase consisting pH 3.2 Monobasic sodium phosphate buffer, Acetonitrile and Ethanol (68:32:02 v/v). All the experiments are carried out in column temperature 40^0C column oven temperature with UV detection at 246 nm. The eluent used for the extraction of the drug consists of pH 3.2 Monobasic sodium phosphate buffer, Acetonitrile and Methanol (50:25:25 v/v). The method is validated for its specificity, linearity, accuracy, precision, Solution stability and robustness. Design expert linear model was applied and a 2^4 full factorial design was applied to evaluate coefficient, ANOVA and also establish the robustness of the method.

KEYWORDS: Budesonide, Liquid Chromatography (LC), Validation, International Conference on Harmonization (ICH), Quality by Design (QbD), ANOVA

I. INTRODUCTION

Budesonide, a second generation glucocorticoid, used in the chronic inflammatory bowel disease, including Crohn's disease and ulcerative colitis.[1,2] Budesonide, a non-halogenated corticosteroid[3], designated chemically chemically as (RS)-11-beta, 16-alpha, 17, 21 – tetrahydroxypregna - l, 4 – diene - 3, 20-dionecyclic 16, 17 - acetal with butyraldehyde. Due to the introduction of the alkyl chain at the C22 atom, budesonide is a mixture of two epimers (22R and 22S) as shown in Figure -1. Both epimers appear to have similar pharmacological effects; however in vitro studies proposed that the R-epimer was two to three times more potent with respect to its anti-inflammatory effects. The empirical formula of budesonide is $C_{25}H_{34}O_6$[4] and its molecular weight is 430.5



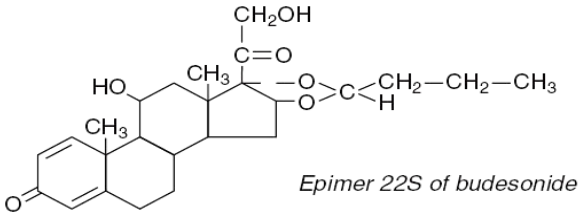


Figure- 1: Budesonide structure

Literature review revealed spectrophotometric[18-20] and chromatographic methods reported for estimation of Budesonide individually[11-16] or in combination with other drugs.[5-10] Only one stability-indicating HPLC method has been reported[17] for the estimation of BDN, the above-mentioned method missed the column temperature.Column temperature plays a mojor role in peak performance,and also the significant influence of % organic phase and flow rate in analysis of BDN were not established. Keeping this view in mind, an improved HPLC method was proposed to investigate the combined effect of buffer pH, % organic phase and flow rate on the chromatographic analysis of BDN.Moreover, there is no method available for the experimental design based evaluation for robustness testing for Budesonide using

HPLC. The aim of the present work was to develop a simple,sensitive, robust and stability[23-24] indicating HPLC method for the estimation of BDS in the formulation dosage form. Experimental design was applied to establish the robustness nature of the method.

2. Experimental

2.1 Materials & Chemicals

Analytical grade reagents are used in method development and validation activity. Budesonide is available as capsules with brand name Budefex was purchased from local market containing Budesonide 3mg.Monobasic sodium phosphate anhydrous, Orthophosphoric acid, Methanol,Ethanol and Acetonitrile are purchased from Merck Chemicals.

2.2 Chromatographic Conditions

HPLC with UV detector used for analysis. Mobile phase consisted of Buffer (4.0 g of Sodium phosphate anhydrous,added 2 ml of phosphoric acid and adjusted pH to 3.2 with diluted Orthophosphoric acid), Methanol and Ethanol (68:32:2 v/v). Thermo Hypersil BDS C18, 150 x 4.6 mm, 3.0 µm stationery phase used as column and a mixture of p H 3.2 buffer,Acetonitrile and Methanol (50:25:25 v/v) used as diluent. The Column oven maintained at 40^0 C with 1.0 ml/min flow rate. The UV detection at 246 nm with injection volume 20µL.The sample time of 25 min.

2.3 Procedure

2.3.1 Preparation of standard solutions

Weighed accurately 90 mg of BDS standard dissolved in 100 mL of diluent .Further transferred accurately 5.0 mL of standard stock solution into a 50 mL diluent to get final concentration of 90 µg/mL of BDS.

2.3.2 Preparation of sample solutions

Weighed and transfer 10 capsules into 200 ml volumetric flask, added 150 ml of diluent, sonicated for 30 min, cool the contents to room temperature and make up with diluent. Filtered the solution through 0.45µ PVDF or 0.45 µ filter. Diluted 15 ml of the above solution to 25 ml and made the volume with diluent.

3. RESULTS AND DISCUSSION

3.1 Method Optimization: The method conditions were evaluated, with aim of ideal peak shape with optimum run time for analyte. Experimental trials were conducted on different parameters such as column, wavelength, buffer composition, mobile phase ratio and flow rate.As BDS eluted as epimers,the resolution between Epimer-B& Epimer-A also have a vital role in the estimation of BDS.Hypersil BDS C18,150 x 4.6 mm, 3.0 µm column provides premier peak shape and finest resolution between epimers. For mobile phase selection, initial trials were taken with different buffers having various pH values. The composition of pH 3.2 buffer(NaH_2PO_4), Acetonitrile and Ethanol (68:32:2 v/v) offers ideal peak shape for each epimer and acceptable resolution between the epimers at column temperature 40°C with a flow rate 1.0 ml/min. The maximum absorbance observed at 246nm.The composition of pH 3.2 buffer, Acetonitrile and Methanol (50:25:25 v/v) used as diluent. The method was finalized in the view of all analytical aspects among the all paths.

3.2 Method validation

3.2.1 Specificity: To establish the non-interference of blank, prepared standard and sample as per test method and injected in to the chromatography system. Chromatogram of blank showed no peak at the retention time of BDS peak. A typical chromatogram of blank and standard were shown in Figure-2 and Figure-3 respectively. Specificity is the capability of the method to quantify the analyte response in its degradation studies. A study was initiated to demonstrate the effective separation of degradents from BDS. The stress conditions and results are reported in Table-1.In all degradation samples, the purity angle found to be less than purity threshold and no purity flag were observed. This indicates that there is no interference from degradents in quantitating the BDS. Significant degradation was observed only in acid degradation. Optimum degradation observed in base,peroxide and Thermal conditions. No significant degradation was observed in Photolytic,water and Humidity. Results of degradation study are given in Tabel-1.Typical chromatograms of degradation samples were shown in Figure-4.

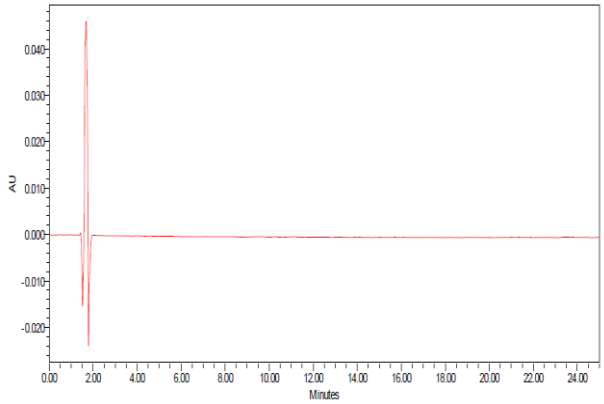


Figure-2: Chromatogram of blank

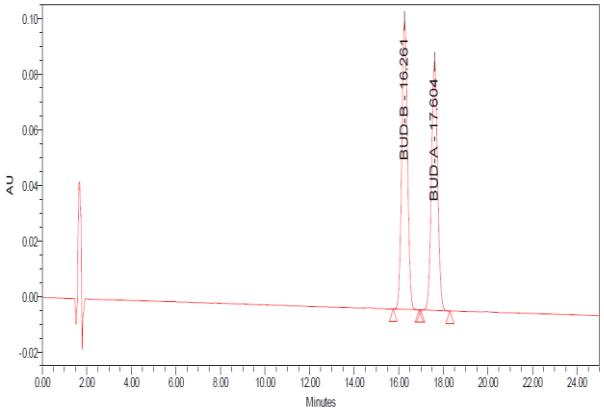


Figure-3: Chromatogram of Standard

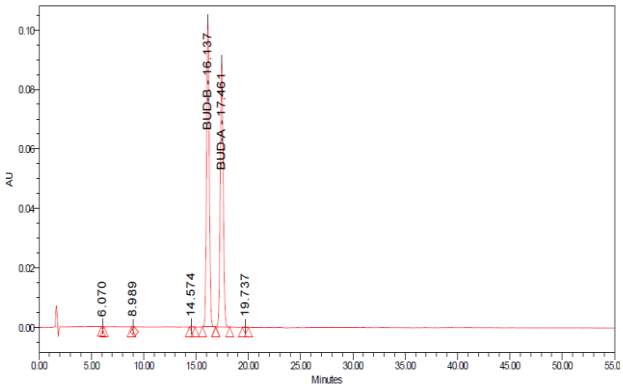
3.2.2 Method Precision

The repeatability of method was evaluated by assaying the six individual sample preparations of BDS.Intermediate precision was established by performing the precision study on different day with different analyst under same analytical conditions. The % RSD was calculated for precision and intermediate precision respectively. Results are reported in Table-2.

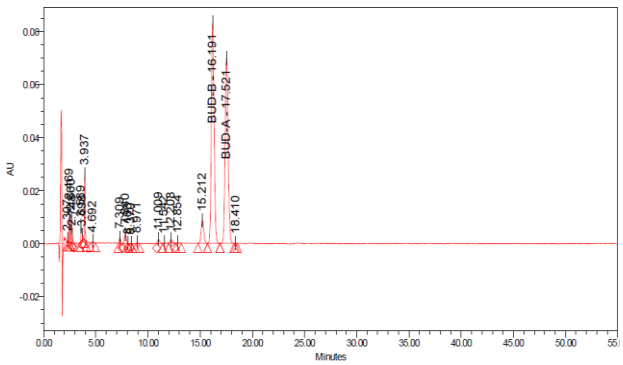
Table-1: Degradation conditions and Results of Interference for BDS from Degradation Products

Name of Degradation	Conditions	Degradation (%)	Purity Angle		Purity Threshold		Purity Flag	
			B[@]	A[#]	B[@]	A[#]	B[@]	A[#]
Unstressed	NA	NA	0.132	0.149	0.341	0.358	No	No
Acid	2N HCl , 12 hours,RT*	19.11	0.221	0.243	0.410	0.452	No	No
Base	0.5N NaOH ,5 min,RT	7.47	0.213	0.203	0.381	0.382	No	No
Peroxide	30% H_2O_2 ,24 hours @ 40° C	6.19	0.213	0.222	0.395	0.394	No	No
Water	Stressed with water for 24 hours at 70° C.	3.10	0.178	0.219	0.346	0.375	No	No
Heat	Stressed at 105° C for 18 hours.	5.78	0.197	0.218	0.387	0.407	No	No
Photolytic	UV light for 200 Watts hr/ square metre	0.88	0.108	0.117	0.281	0.290	No	No
	Visible light for 1.2 million lux hours							
Humidity	Exposed to humidity at 90% RH for 10 days	0.56	0.114	0.130	0.281	0.294	No	No

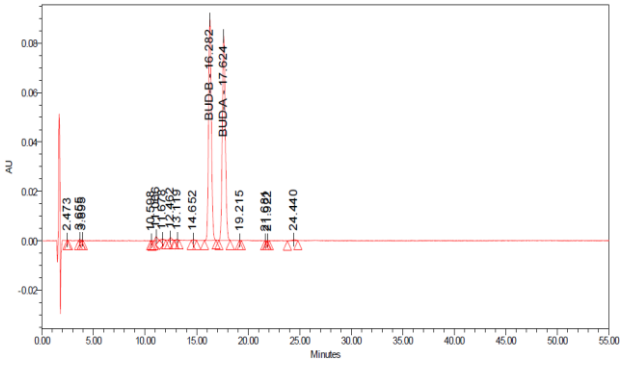
**RT-Room Temperature, [@]Budesonide Epimer B ,[#]Budesonide Epimer A*



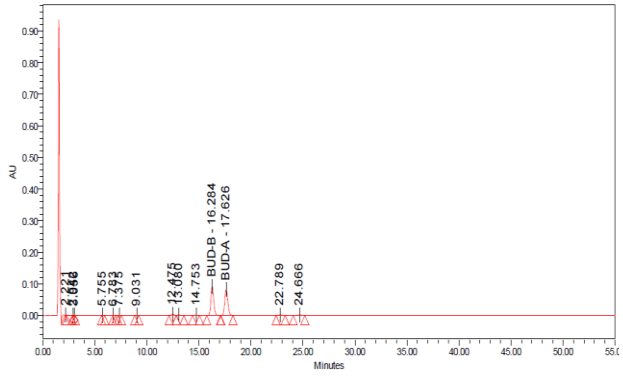
(a) unstressed sample



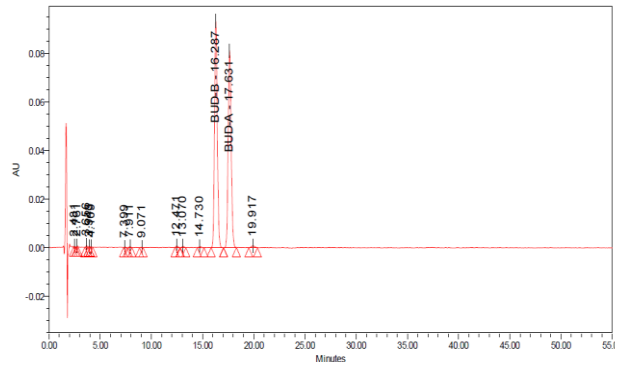
(b) Acid stressed sample



(c) Base stressed sample



(d) Proxide stressed sample



(e) Thermal stressed sample

Figure-4: Typical chromatograms of Degradation samples: (a) unstressed sample (b) Acid stressed sample (c) Base stressed sample (d) Peroxide stressed sample (e) Thermal stressed sample

3.2.3 Linearity: Linearity was established by plotting a graph between concentrations versus peak area responses of BDS and determined the correlation coefficient. A series of solutions of BDS were prepared in the concentration of range from 44.9545µg/mL to 134.8634 µg/mL and analyzed as per test method. A graph was plotted to concentration in µg/mL on X-axis versus peak area on Y-axis and calculated correlation coefficient.The results are summarized in the Table -2.

Table-2: Method precision, intermediate precision, linearity and accuracy data of BDS

Parameter			Results
Precision(n=6) ,%Mean assay (%RSD)			100.7 (0.4)
Intermediate precision(n=6) ,%Mean assay (%RSD)			100.7 (0.3)
Linearity range(µgmL$^{-1)}$			45.9545µg/ml-134.8634µg/ml
Correlation coefficient			0.999
Slope			39626.74
Intercept			-6417.2478
Accuracy	50%	% Mean recovery	98.8
	100%		99.0
	150%		98.8

3.2.4 Accuracy: Accuracy was established by determination of recovery at 50%, 100% and 150% of target test concentration. Three replicates were prepared and analyzed for 100% level. Performed six replicates for lower and higher concentrations (50% and 150%) and analyzed as per test method. The percent recovery was calculated and results reported in Table-2.The % recovery for each level was found to be within the limit of 95%-105% indicates the method was more accurate for intended use.

3.2.5 Solution stability

3.2.5.1 Stability of sample and standard: The stability of BDS in test solution was conducted in room temperature at Initial, after 1 day, after 2 days, 7 days. The % assay of BDS in test preparation was estimated against a freshly prepared standard at each time and also to establish the stability of BDS in standard solution in room temperature was conducted at Initial, after 1 day, after 2 days and after 7 days. The similarity factor for standard preparation was estimated against a freshly prepared standard at each time. Results are reported in Table-3. From this study, it was concluded that the both standard and samples are stable for 7 days in room temperature.

Table-3: Results of sample and standard solution stability of BDS

Sample solution stability						Standard Similarity factor	Mobile phase Stability		
Condition	Time in days	Test-1 %Assay	Test-2 %Assay	%Difference from Initial			a	b	c
				Test-1	Test-2				
Room temperature	Initial	100.4	101.4	NA	NA	NA	1.0	2.7	19456
	Day 1	100.4	100.9	0	0.5	1.00	1.1	2.7	19209
	Day 2	100.1	101.1	0.3	0.3	1.00	1.1	2.7	18899
	Day 7	98.6	99.7	1.8	1.7	0.99	1.1	2.7	18617

(a)Tailing factor for Budesonide Epimer –B peak in standard injection.(b)Resolution between Budesonide Epimer peak-B and Epimer peak-A.(c)Plate count for Budesonide Epimer –B peak in standard injection.

3.2.5.2 Stability of Mobile phase: To establish stability of mobile phase in room temperature at initial, after 1 day, after 2 days and after 7 days was conducted. The system suitability parameters were evaluated as per the test method and found to be within the limits. From this study, it was established that the mobile phase is stable for a period of 7 days in room temperature .The results are summarized in Table-3.

3.2.5.3 Filter validation: To demonstrate the suitability of filters was established using 0.45µm PVDF filter and 0.45µm Nylon filter. Test preparations were prepared in duplicate and these solutions were centrifuged and filtered and injected in chromatographic system. Calculated % assay for centrifuged and filtered test solutions against unfiltered standard. The difference in % assay between centrifuged and filtered test solutions was found to be within the limits. From the above study it was established that both filters Nylon and PVDF are suitable for filtration. The results are summarized in Table-4

Table-4: Results of filter validation samples data

% Assay					
Sample No.	Centrifuged	Nylon filtered	Difference	PVDF filtered	Difference
1	98.7	98.8	0.1	98.9	0.2
2	98.5	98.6	0.1	98.8	0.3

3.2.5 Robustness: As defined by ICH, robustness study was performed to establish the potentiality of method for slight changes in the method conditions like flow, Acetonitrile composition in mobile phase,pH of buffer in mobile phase and column oven temperature.In the Acetonitrile composition in mobile phase,mobile phase

was prepared having 90% and 110% of the method organic phase (Acetonitrile) composition. Standard solution prepared as per test method and injected into chromatographic system. The System suitability parameters were evaluated with above mobile phases. It was found that the results are within the limits for mobile phase with 110% of Acetonitrile, whereas in mobile phase with 90% of Acetonitrile, retention time of BDS was found to be exceeding the run time. Hence study was repeated by preparing mobile phase with 95% of Acetonitrile and the results were found to be within limits. No significant effect was observed on system suitability parameters like plate count,resolution and tailing factor. The results were shown in Table-5.In all the above variable conditions the system suitability parameters are found within the limit.

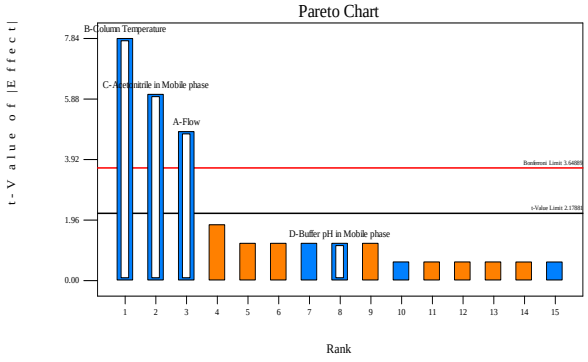
Table-5: System suitability parameters of robustness conditions

Parameter	% RSD (≤ 2%)	Tailing factor (≤ 1.5)	Plate count (>3000)	Resolution (≥ 1.5)
System suitability	0.1	1.0	18599	2.7
Flow-0.8 ml min^{-1}	0.1	1.1	18563	2.7
Flow-1.2 ml min^{-1}	0.3	1.1	18571	2.7
Column temperature 35^0C	0.1	1.1	18746	2.8
Column temperature 45^0C	0.1	1.1	18289	2.5
Acetonitrile composition (110%)	0.1	1.1	18951	2.5
Acetonitrile composition (95%)	0.2	1.1	17990	2.7
pH of buffer (3.0)	0.1	1.0	16971	2.5
pH of buffer (3.4)	0.1	1.0	16924	2.5

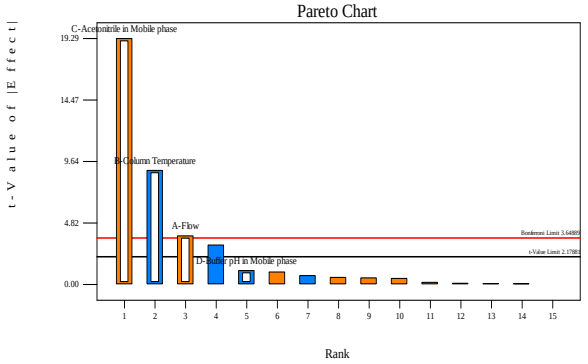
3.2.5 Experimental Design Approach: Design expert software (Stat-Ease Inc, Statistics made easy, Minneapolis, MN, USA, Version 9.0) was used for experimental design study. The full factorial design needs less dimensions than the traditional one-at-a-time observation to provide same precision. At the same time, it offers the information of interfaces between the factors. In order to create the real-time difference of the desired factors on the measured responses, an approach using experimental design is used in robustness testing.[25-26] And also an analytical method is fast and it requires few factors, a prominent choice for robustness testing may be design expert, generally used because of its high competence with respect to lesser number of runs required in the full factorial model. In order to study the four variables at two levels, a 2^4 full factorial design the design used in robustness testing of BDS. Plate count and resolution is measured as responses. ANOVA with a linear model was employed to determine the model coefficients and also check the robustness of the method.

Four factors and two levels caused full factorial design, in addition to that, two center points resulted total 18 experiments points-those are carried out in random order. The effects of the four factors in the plate count and resolution of BDS are shown in a Pareto chart (Figure 5 (a), 5 (b)). The effect of independent factors on the dependent factors was explained by Perturbation plots . Figure 5(c) is related to the effect of independent factors on Resolution between epimer-B and epimer-A.It shows that there is no effect of pH buffer (D) on Resolution , but the Column temperature (B) shows an incredible effect on resolution as the increase in the column temperature decreases. Figure 5(d) gives the information about the effect of independent factors on plate count. This figure shows that there is no effect of p H of mobile phase on plate count, but acetonitrile composition in mobile phase plays an vital role on the same as when the acetonitrile composition increases, the plate count also increases.

Four factors were considered: flow rate mL/min (A), column temperature (B), Acetonitrile composition % in mobile phase (C), and pH of buffer (D). The factors and responses for the trials are shown in Table -6.



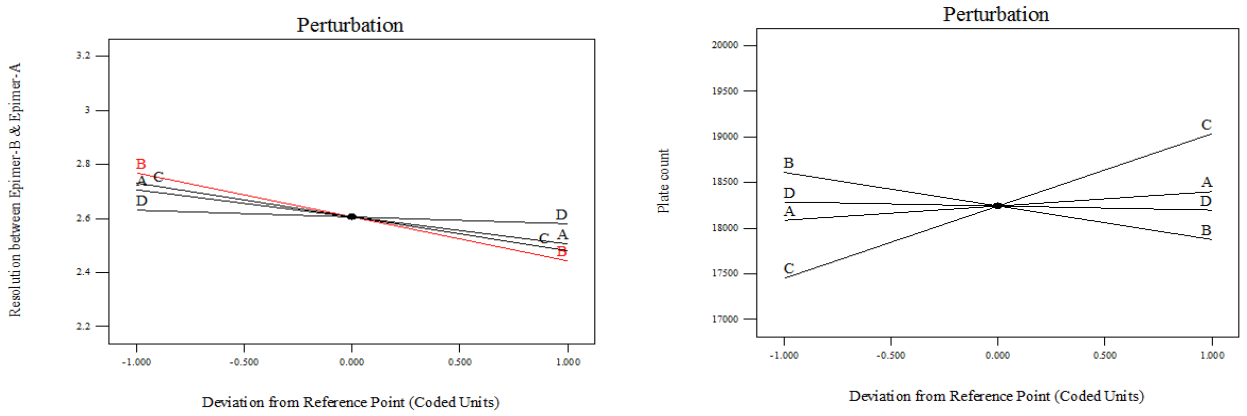
(a)



(b)

Figure-5 : Pareto chart effect of factor (a) B >C >A>D in Resolution (b) C >B >A>D in Plate count

Standard and sample prepared and injected into chromatograph system. The resolution and plate count was studied as responses.The statistical ANOVA was employed to create the significant and most contributing factors where they were ordered on the basis of the degree of F-ratio. The higher the F-value corresponds with the smaller "Prob>F" value, the more significant the resultant model.[27] Table-7 and Table-8 are showing the reading of the ANOVA analysis for the Resolution and plate count, where the F-value and P-value of the model were 31.49 and 0.0001& 49.05 and 0.0001 respectively, demonstrating that the projected model fits the experimental data satisfactorily. ANOVA analysis data also concluded that, the pH of the mobile phase did not shows any effect on the response.



(c) **(d)**

Figure 5(c) :Perturbation plot of Resolution of Epimer-B and Epimer-A and Figure 5(d) Plate count as a responces against factors flow rate (A), Column temperature (B) and Acetonitrile composition (C) pH of buffer in mobile phase

Table-6: The levels and range of the variables in the 2^4 full factorial design

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response
Std	Run	A:Flow	B:Column	C:Acetonitrile in	D:Buffer pH in	Resolution between	Plate
		ml/min	deg C	%			
15	1	0.8	45	35.2	3.4	2.3	18367
8	2	1.2	45	35.2	3	2.2	18659
9	3	0.8	35	30.4	3.4	2.9	17434
17	4	1	40	32.8	3.2	2.7	18764
11	5	0.8	45	30.4	3.4	2.6	17005
12	6	1.2	45	30.4	3.4	2.5	17209
6	7	1.2	35	35.2	3	2.6	19760
3	8	0.8	45	30.4	3	2.8	17054
18	9	1	40	32.8	3.2	2.6	18678
13	10	0.8	35	35.2	3.4	2.8	19241
5	11	0.8	35	35.2	3	2.7	19347
14	12	1.2	35	35.2	3.4	2.5	19533
1	13	0.8	35	30.4	3	3.1	17543
7	14	0.8	45	35.2	3	2.4	18233
4	15	1.2	45	30.4	3	2.4	17342
2	16	1.2	35	30.4	3	2.8	17896
16	17	1.2	45	35.2	3.4	2.3	18669
10	18	1.2	35	30.4	3.4	2.7	17658

Table 7: ANOVA for 2^4 full factorial design for Response :Resolution between Epimer-B & Epimer-A

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	0.84	4	0.21	31.49	< 0.0001	significant
A-Flow	*0.16*	*1*	*0.16*	*23.92*	*0.0003*	
B-Column Temperature	*0.42*	*1*	*0.42*	*63.17*	*< 0.0001*	
C-Acetonitrile in Mobile phase	*0.25*	*1*	*0.25*	*37.38*	*< 0.0001*	
D-Buffer pH in Mobile phase	*0.010*	*1*	*0.010*	*1.50*	*0.2431*	
Residual	0.087	13	6.688E-003			
Lack of Fit	*0.082*	*12*	*6.829E-003*	*1.37*	*0.5911*	*not significant*
Pure Error	*5.000E-003*	*1*	*5.000E-003*			
Cor Total	0.93	17				

Df: degree of freedom, Cor Total: corrected total sum of squares, Values of Prob> F less than 0.05 reprasents that the models are significant

Table 8: ANOVA for 2^4 full factorial design for Response: Plate count

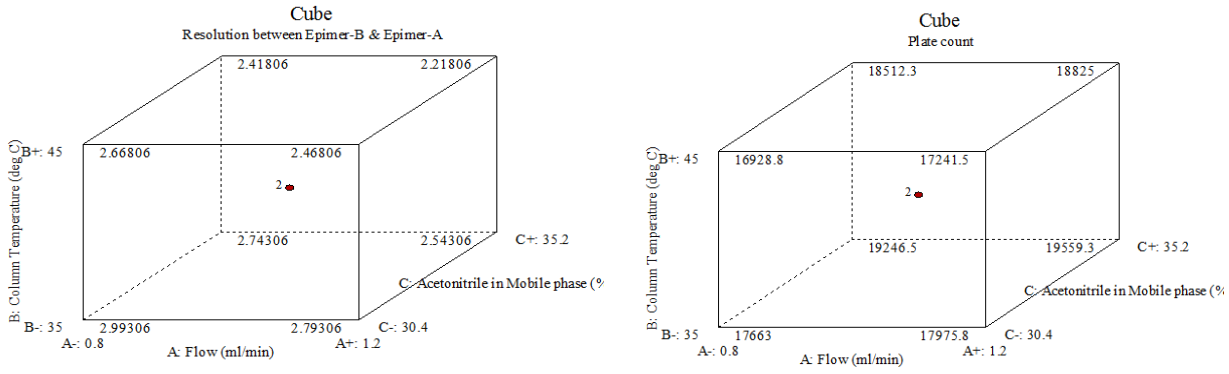
	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	1.261E+007	4	3.152E+006	49.05	< 0.0001	significant
A-Flow	*3.913E+005*	*1*	*3.913E+005*	*6.09*	*0.0283*	
B-Column Temperature	*2.156E+006*	*1*	*2.156E+006*	*33.56*	*< 0.0001*	
C-Acetonitrile in Mobile phase	*1.003E+007*	*1*	*1.003E+007*	*156.07*	*< 0.0001*	
D-Buffer pH in Mobile phase	*32220.25*	*1*	*32220.25*	*0.50*	*0.4914*	
Residual	8.355E+005	13	64265.40			
Lack of Fit	*8.318E+005*	*12*	*69312.69*	*18.74*	*0.1788*	*not significant*
Pure Error	*3698.00*	*1*	*3698.00*			
Cor Total	1.345E+007	17				

R^2 is the measure of the amount of deviation around the mean described by the model.When R^2 equals 1, the relationship is perfect and all values of the variables lie on a straight line. Adjusted R^2 calculates the proportion of the variation in the dependent variable accounted by the instructive variables. R^2 increases with the addition of a new term to a model, but if the new term improves the model, then adjusted R^2 increases. The adjusted R^2 can be negative, but it will be $\leq R^2$. The predicted R^2 explains a predicted response of a regression model a observation. These statistics are less capable of providing valid predictions for a new model. Adjusted R^2, predicted R^2 can be negative and it is always less than R^2. As reported in Table- 9 the R^2 value indicates that 90 % of the data variability was effectively explained by the model.

Table 9:The regression equation obtained for resolution and plate count for coded factors, along with the parameters of regression are tabulated below:

Response	Regression equation for coded factors	R2	Predicted R2	Adjusted R2
Resolution	+2.61-0.10*A-0.16*B-0.13*C-0.025*D	0.9065	0.8152	0.8777
Plate count	+18244.00+156.37*A-367.13*B+791.75*C-44.88*D	0.9379	0.9037	0.9187

The above information concludes that with a slight change in flow rate, column temperature,% acetonitrile composition in the mobile phase during analysis, the resolution and plate count will not be affected. Hence, from this data generated by the model, it can be illustrated that the resolution decreases with an increase in column temperature, and also the plate count increases with an increase in acetonitrile composition in mobile phase shown in the cubical representation of the responses in Figure-6.



(a) **(b)**

Figure 6. Three-dimensional cubical representation of the full factorial for the predicted response (a) Resolution of BDS plotted on the y–axis as a function of factor A (flow rate), B (column temperature) and C (% acetonitrile in mobile phase); fixed factor: D (pH of buffer); (b) Plate count of BDS plotted on the y–axis as a function of factor A (flow rate), B (column temperature) and C (% acetonitrile in mobile phase); fixed factor: D (pH of buffer);

4. CONCLUSION

The experimental design concludes explains the intractions of the crucial method components including column temperature, mobile phase flow rate,% acetonitrile composition and pH of the buffer in the mobile phase. The interrelationships were studied and the preliminary enhanced conditions were attained for each combination. Here, a better understanding of the factors influencing chromatographic separation and greater confidence in the capacity of the methods to meet their intended purposes is stablished. A gradient RP-HPLC method was developed successfully for the determination of Budesonide pharmaceutical dosage formulation. The method results have proven that the established method is selective, precise, accurate, linear, robust, filter compatible, and stability-indicating. Forced degradation data showed that the proposed method is specific for the analytes and free from the interference of degradation products. Moreover, it may be applied for the study of content uniformity and in vitro release test profiling of Budesonide pharmaceutical dosage forms.

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