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Development and Validation of Advanced UV-Spectrophotometric Methods and a RP-HPLC Method for the Simultaneous Estimation of Beclomethasone Dipropionate and Formoterol Fumarate Dihydrate in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

The presented research work aims to develop and validate UV-Spectrophotometric as well as RP-HPLC methods for the simultaneous estimation of Beclomethasone Dipropionate (BDP) and Formoterol Fumarate Dihydrate (FFD). These methods offer a higher degree of sensitivity than the already present methods of analysis. By implementing advanced spectroscopic techniques such as Dual Wavelength Method and First Derivative Spectroscopy it is found that the sensitivity of the methods is increased. The linearity of both the methods was in the range of 10µg/ml to 50µg/ml for BDP and 1µg/ml to 5µg/ml for FFD, with an r^2 value of 0.999 and 0.9988 respectively for Dual Wavelength Method and First Derivative Method. The LOD values of Dual Wavelength Method and First Derivative Method were found to be 0.127µg/ml and 0.016µg/ml respectively. A RP-HPLC Method has also been developed and validated for this combination having a linearity in range of 50µg/ml to 250µg/ml for BDP and 1.5µg/ml to 7.5µg/ml for FFD. The r^2 value of BDP and FFD was found to be 0.9995 and 0.994. The application of this RP-HPLC Method may also be extended for the simultaneous estimation of a triple combination of Beclomethasone Dipropionate, Formoterol Fumarate and Glycopyrronium Bromide (GPB).

Keywords: Beclomethasone, Formoterol, Glycopyrrolate, Dual-Wavelength-Method, First-Derivative, Spectroscopy, RP-HPLC

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INTRODUCTION

A Combination of Beclomethasone Dipropionate and Formoterol Fumarate Dihydrate is used to treat COPD and other lung disorders. Beclomethasone here as a corticosteroid increases the expression of β2 – adrenoceptors and protects them against down-regulation. Conversely, β2 – Adrenergic receptor agonists improve the anti-inflammatory action of corticosteroids. Thus this combination synergistically relaxed human bronchi; the extent of such an interaction is very strong at low-to-medium concentrations in the small airways. [1]

Various methods for the analysis of this combination have already been reported such as; Simultaneous estimation of Beclomethasone Dipropionate and Formoterol Fumarate in Rotacap Dosage form using UV-Spectroscopic Method [2], RP-HPLC method development and validation of Beclomethasone and Formoterol [3], HPLC determination of Beclomethasone and its degradation products [4], Comparison of Beclomethasone / Formoterol versus Budesonide / Formoterol combination therapy in asthma. [5]

Beclomethasone Dipropionate is chemically 9α-chloro-11β-hydroxy-16β-methyl-3, 20-dioxopregna-1, 4-diene-17, 21-diyldipropionate and Formoterol Fumarate is a dihydrate salt of fumaric acid with (RS)-2′-hydroxy-5′-[(RS)-1-hydroxy-2-[[(RS)-p-methoxy-α-methylphenethyl] amino] ethyl] formanilide. The structure of Beclomethasone Dipropionate and Formoterol Fumarate are as represented below in **Figure 1** and **Figure 2**.

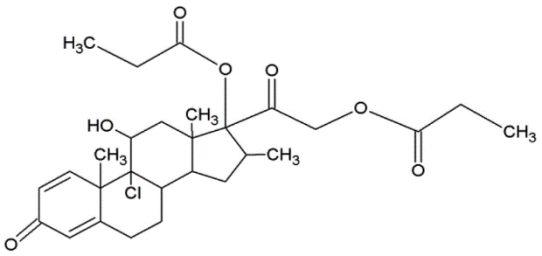

Figure 1: Structure of Beclomethasone Dipropionate

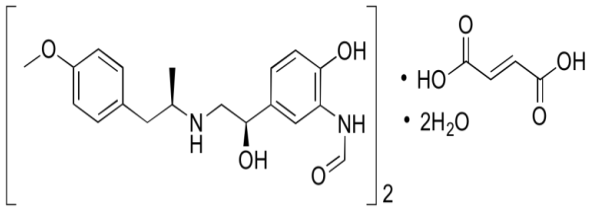

Figure 2: Structure of Formoterol Fumarate

MATERIALS AND METHOD

Instruments / Apparatus / Softwares:

Shimadzu HPLC System equipped with UV Detector, UV-Visible Spectrophotometer, pH meter and electronic balance.

Chromatographic Condition for RP-HPLC:

ANALYTICAL – C_{18} HyperChrome ODS-BP Column was used having dimensions of 250mm x 6mm, and an internal diameter of 5µm. The optimized mobile phase used was ACN: Ammonium Acetate Buffer (70:30). A flow rate of 1 ml/min was set and the wavelength of detection was set to 218nm for the detection of BDP and FFD, whereas for the detection of the triple combination of BDP, FFD and GPB the wavelength used was 252nm. A fixed injection volume of 20µL was taken each time and the run time was set to 15 minutes.

Preparation of Standard Stock Solution:

For UV-Spectroscopic methods, the standard stock solutions of BDP as well as FFD are prepared by taking 10mg of the standard drug in 10ml Methanol to get a concentration of 1000µg/ml.

For RP-HPLC Method, weigh accurately an amount of 10mg of Beclomethasone Dipropionate and transfer it into a previously calibrated volumetric flask. The volume is made up to the mark using the mobile phase itself. Similarly weigh accurately an amount of 10mg of Formoterol Fumarate and transfer it into a previously calibrated volumetric flask. The volume is made up to the mark using the mobile phase itself.

Preparation of working standard solution:

For UV-Spectroscopic methods, the working stock solution of BDP is prepared by taking 2ml of the standard stock solution and transferring it to a 10ml volumetric flask. The volume is made up to the mark using Methanol to get a concentration of 200µg/ml. Similarly the working standard solution of FFD is prepared by taking 1ml of the standard stock solution and transferring it to a 10ml volumetric flask. The volume is made up to the mark using Methanol to get a concentration of 100µg/ml.

For RP-HPLC Method the working standard solution of BDP is the Standard Stock Solution itself, whereas for FFD the working standard solution must be prepared. The Working Standard Solution of FFD is prepared by taking 1ml of the Standard Stock solution and diluting this solution up to 10ml using the mobile phase.

Derivative Spectroscopy:

Derivative Spectroscopy involves the conversion of a normal spectrum (fundamental, zero-order spectrum) to its first, second or higher derivative spectra by differentiating absorbance of the sample with respect to wavelength (λ). The differentiation of zero-order spectrum can lead to separation of overlapped signals, elimination of background caused by presence of other compounds in a sample, improvement of resolution of mixtures as it enhances the detectability of minor spectral features, and enhancement of sensitivity and specificity. [6]

Dual Wavelength Method:

Dual wavelength method also known as two wavelengths method, facilitates analyzing a component in presence of an interfering component by measuring the absorbance difference (ΔA) between two points in the mixture spectrum. In this method one of the drugs is considered as a component of interest and the other drug is considered as an interfering component and vice-versa. The basis for such method is the selection of two wavelengths where the interfering component shows the same absorbance (ΔA equals zero) whereas the component of interest shows significant difference in absorbance with concentration. ΔA between two points on the mixture spectra is directly proportional to the concentration of the component of interest independent of interfering component. [6]

Method Validation Parameters: [7]

The developed UV-Spectroscopic and RP-HPLC methods were validated in accordance with the current ICH guidelines - Q2 (R1). The parameters which were validated include Specificity, Linearity, Range, Limit of detection, Limit of quantification, Precision, Accuracy, Ruggedness, Robustness as well as system suitability tests for RP-HPLC.

Specificity:

Specificity is one of the first validation parameters that need to be studied. It represents the ability of the analytical method to generate signals which are free from interferences. It is the capability of the method to estimate precisely an analyte in the presence of interferences that may be expected to be present in the sample. Typically these interferences may be degradants, matrix or impurities present in the sample. Identification tests should be able to differentiate the compound of closely related structures which are expected to be present.

Linearity and Range:

Linearity of an analytical method represents that there is a linear relationship between the signal and the concentration of the analyte under investigation. Linearity should be evaluated by visual inspection of a plot of signals as a function of the analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. It can be analyzed by performing single measurements at several different analyte concentrations. The data is then processed using a linear least square regression method. The resulting plot gives us the intercept, slope and correlation coefficient values which is able to provide the desired information on the linearity of the method.

Precision:

The precision of an analytical method represents the closeness of individual measurements to each other under similar analytical condition and it is divided in three categories. The ICH guideline – Q2(R1) suggest that repeatability should be assessed using a minimum of 9 determination covering the specified range for the procedure (3 concentrations / 3replicates each) or a minimum of 6 determinations at 100% of the test concentration.

- Intra-day Precision: It can be defined as within-day precision and performed by 3 replicates at 3 different concentrations.
- Inter-day Precision: It can also be referred to as between-day precision. It is determined by 3 replicates at 3 different concentrations.
- Repeatability: Precision which is measured keeping the same analyst and same operating condition over short period of time?

Accuracy:

Accuracy is defined as a measure of closeness of agreement between the experimental value and the accepted reference value. It is one of the most important parameters which is to be considered in the validation of an analytical method. It can also be defined as the percent recovery of known amounts of standard drug added to a sample. There are three way to determine accuracy:

- Recovery of analyte spiked into blank matrix
- Comparison to a reference standard
- Standard addition of the analyte

Limit of Detection (LOD):

Limit of detection is determined by the analysis of samples with known concentration of drug and by establishing that minimum level at which the analyte can detected, but not necessarily quantitated as a precise value. LOD is generally expressed in terms of concentration of analyte in the sample. A number of approaches may be applied according to the ICH for determination of the detection limit of a sample depending on the nature of the analyte, suitability of the method and instrument which is used for analysis. The acceptable approaches are Signal to noise ratio, Standard deviation of the slope of the linearity plot, Visual evaluation, Standard deviation of the response.

The formula for calculating the LOD is:

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

Where, σ = the standard deviation of the responses

S = the slope of the calibration curve

Limit of Quantification (LOQ):

Limit of quantification is defined as the least amount of concentration of the analyte in a sample which can be estimated with appropriate accuracy and precision under the affirmed experimental conditions.

The formula for calculating LOQ is:

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

Where, σ = the standard deviation of the responses

S = the slope of the calibration curve

Robustness:

Robustness of an analytical method can be defined as the ability of the analytical method to remain unaffected by small but deliberate variation in the critical method parameters. The capability to reproduce the results of the analytical method under different circumstances without the occurrence of unforeseen differences is known as robustness. The method parameters which may be varied in RP-HPLC Method include pH, flow rate, column temperature, mobile phase composition, solvents grades, detection wavelength and percentage ACN.

System Suitability Tests (SST):

These tests are to prove that the system is working ideally before the analysis on the HPLC analyser is carried out. SSTs are required to be done before every sample analysis. The System Suitability Testing (SST) limits should confirm to the guidelines provided by the Center for Drug Evaluation and Research (CDER) as well as the International Conference on Harmonization (ICH). The parameters which are needed to be checked in SST are retention time, theoretical plate number, tailing factor, column efficiency, signal-to-noise ratio and resolution.

RESULTS AND DISCUSSION

Linearity:

In each of the developed methods 4 sets of calibration curves were plotted between the absorbance and concentration (in case of UV-Spectrophotometric Methods), or area and concentration (in case of RP-HPLC Method). The Calibration Curve that showed the best r^2 values is represented below in **Figure 3-6** for each of the four methods. The linearity of First order derivative spectroscopy was taken by first taking the zero order spectra of both drugs individually in the range of 200-400nm (**Figure 3**), and then converting them to the first derivative spectra. The transformation of zero order spectra to first derivative spectra was done in the software using a Δ λ = 5nm and scaling factor of 20. Thus, here at 239nm (Zero Crossing Point of BDP) the estimation of FFD is possible, whereas at 268.4nm (Zero Crossing Point of FFD) the estimation of BDP is done as shown in **Figure 4.** The linearity of the First Order Derivative Method was found in the range of 10 µg/ml to 50 µg/ml for BDP (r^2 = 0.9992) and 1 µg/ml to 5 µg/ml for FFD (r^2 = 0.9989).

In case of Dual wavelength method at 215nm and 266nm the absorbance values of BDP are the same, but the absorbance values of FFD at these two wavelengths are different. This difference may be used for the quantification of FFD. Similarly at wavelengths 259nm and 284nm the absorbance values of FFD are the same, but the absorbance values of BDP at these two wavelengths are different. This difference may be used for the quantification of BDP. The linearity as shown in **Figure 5** of Dual Wavelength Method was found in the range of 10 µg/ml to 50 µg/ml for BDP (r^2 = 0.999) and 1 µg/ml to 5 µg/ml for FFD (r^2 = 0.9988).

The linearity of the developed RP-HPLC Method of BDP and FFD was established using the optimized mobile phase of ACN: Ammonium Acetate Buffer with a pH set to 4.5 as shown in **Figure 6**. The concentration range in which the beer-lambert law was followed was found to be 50 µg/ml to 250 µg/ml for BDP and 1.5 µg/ml to 7.5 µg/ml for FFD. The r^2 value of RP-HPLC linearity was found to be 0.9995 for BDP and 0.9994 for FFD.

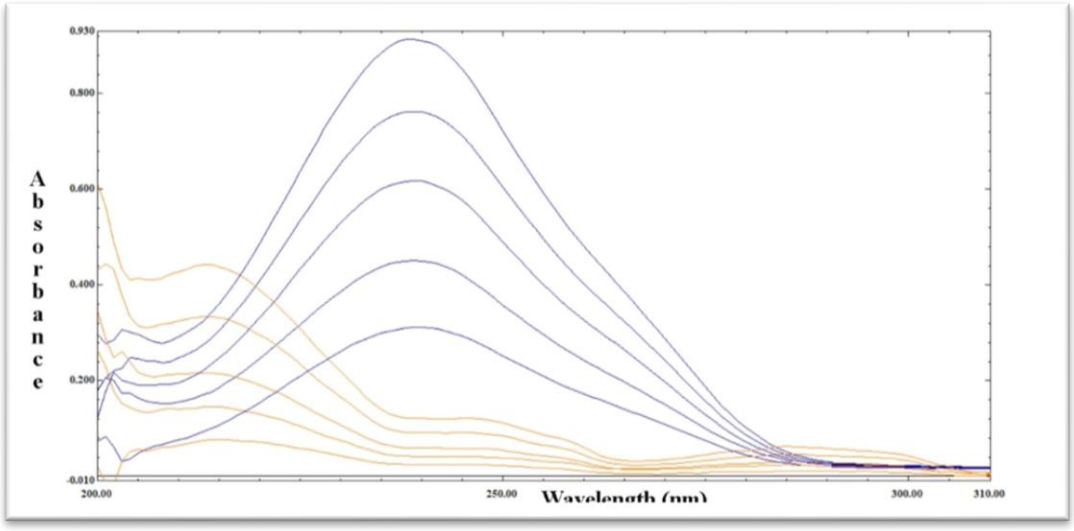

Figure 3: Zero order overlay of BDP and FFD

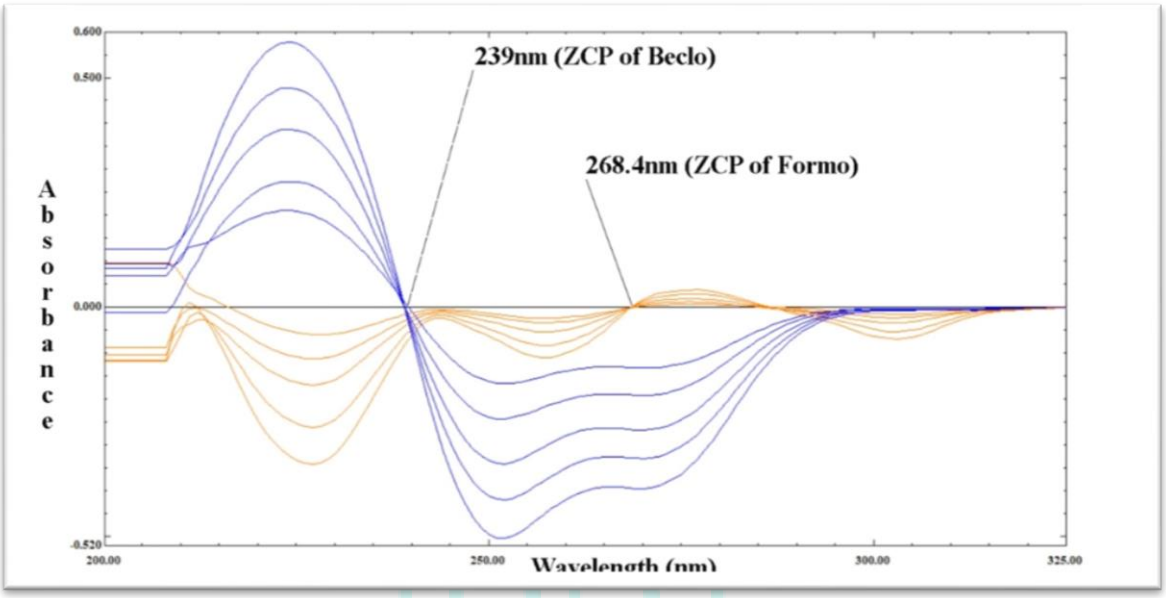

Figure 4: First Derivative overlay spectra of BDP and FFD

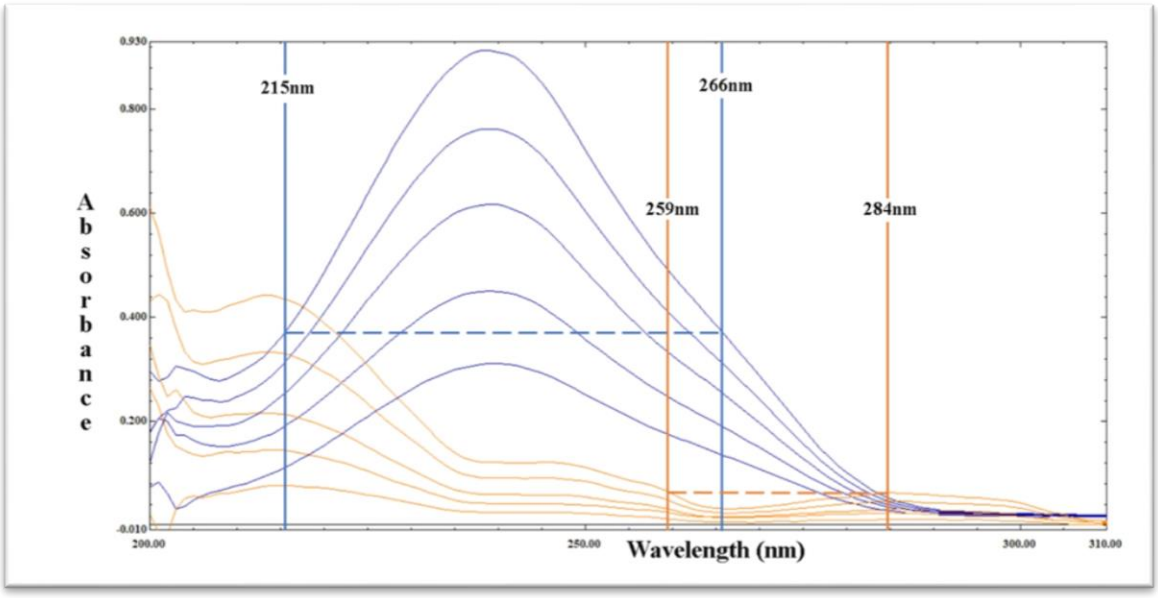

Figure 5: Dual Wavelength Spectra of BDP and FFD

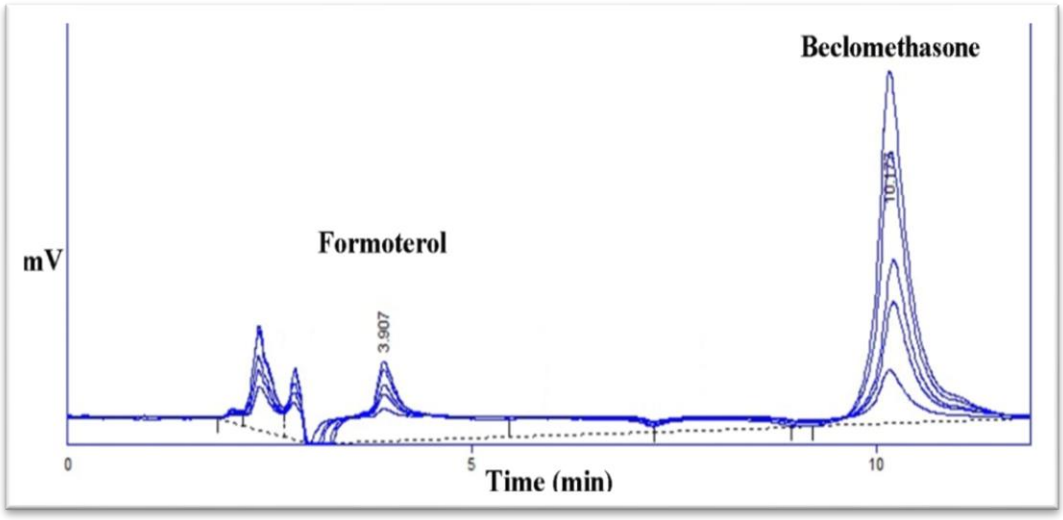

Figure 6: RP-HPLC Overlay for BDP and FFD

Limit of Detection and Limit of Quantification:

The Limit of Detection and Limit of Quantification was calculated using the above formulas and the data is represented in **Table 1** below.

Table 1: Limit of Detection and Limit of Quantification of BDP and FFD

Drug	Limit of Detection (LOD)			Limit of Quantification (LOQ)		
	First Derivative Method	Dual Wavelength Method	RP - HPLC Method	First Derivative Method	Dual Wavelength Method	RP - HPLC Method
BDP	0.739µg/ml	0.127 µg/ml	3.283µg/ml	2.240µg/ml	0.384 µg/ml	9.951µg/ml
FFD	0.183µg/ml	0.016 µg/ml	0.081µg/ml	0.556µg/ml	0.049 µg/ml	0.248µg/ml

Precision:

The precision of an analytical method expresses the closeness of agreement between a series of measurements which are obtained by performing multiple samplings of the same homogenous sample under the given conditions of the method.

In this research paper, various methods have been analysed for precision at two levels:

1. Repeatability (precision under the same operating conditions over a short interval of time)
2. Intermediate precision (variations in the results obtained at different intervals)

From the results of precision it may be concluded that both the methods developed are precise as the %RSD values are less than 2. It also may be concluded here that the Dual Wavelength Method is more precise than the 1st Derivative Method.

In the data represented below **Table 2** represents the repeatability data, whereas **Table 3** and **Table 4** present the data for intra-day and inter-day precision respectively. Precision data are represented in terms of %RSD and the nominal concentration of Beclomethasone Dipropionate (BDP) and Formoterol Fumarate Dihydrate (FFD) was kept 50 µg/ml and 1.5 µg/ml respectively for UV-Spectrophotometric Methods and 200 µg/ml (BDP), 6 µg/ml (FFD) for RP-HPLC Method.

Table 2: Repeatability data expressed in terms of % RSD

Repeatability							
Beclomethasone				Formoterol			
Parameter	1st Der.	DWM	RP-HPLC	Parameter	1st Der.	DWM	RP-HPLC
Mean	0.396	0.446	2343.452	Mean	0.020	0.081	183.422
SD	0.00173	0.00057	11.82	SD	0.00037	0.00074	1.794
% RSD	**0.437**	**0.239**	**0.504**	**% RSD**	**1.788**	**0.912**	**0.978**

Table 3: Intra-day data expressed in terms of % RSD

Intra-Day Precision							
Beclomethasone				**Formoterol**			
Parameter	1st Der.	DWM	RP-HPLC	Parameter	1st Der.	DWM	RP-HPLC
Mean	0.396	0.0603	2351.307	Mean	0.0208	0.0818	182.598
SD	0.00221	0.00047	7.33	SD	0.00037	0.00068	2.54
% RSD	**0.559**	**0.382**	**0.311**	**% RSD**	**1.788**	**0.839**	**1.394**

Table 4: Inter-day data expressed in terms of % RSD

Inter-Day Precision							
Beclomethasone				**Formoterol**			
Parameter	1st Der.	DWM	RP-HPLC	Parameter	1st Der.	DWM	RP-HPLC
Mean	0.395	0.06	2347.635	Mean	0.0208	0.0808	183.654
SD	0.00197	0.00037	17.05	SD	0.00037	0.00068	1.8
% RSD	**0.498**	**0.619**	**0.726**	**% RSD**	**1.788**	**0.85**	**0.984**

Accuracy:

Recovery studies for the UV-Spectrophotometric methods were conducted using the Standard Addition Method by taking a nominal concentration of 20µg/ml for Beclomethasone Dipropionate and 0.6µg/ml for Formoterol Fumarate Dihydrate from the formulation (test sample) and then spiking this solution by 80%, 100% and 120% of standard drug (API).

It can be inferred from the presented data that all the developed methods are accurate according to the ICH Guidelines. It can also be concluded here that the Dual Wavelength Method (presented in **Table 6**) is more accurate than the 1st Derivative ZCP Method (presented in **Table 5**) as the values of % Recovery are closer to 100%.

The recovery studies of the RP-HPLC Method were also carried out using the Standard Addition Method wherein the nominal concentration of BDP was 100 µg/ml, and FFD was 3 µg/ml. The accuracy data of this method is represented in **Table 7** below.

Table 5: Accuracy of First Derivative Method expressed in terms of % Recovery

First Derivative Method					
Beclomethasone Dipropionate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Conc. Recovered	**%Recovery ± SD (n=3)**
1	80	20 µg/ml	16 µg/ml	35.84 µg/ml	99.557 ± 0.00205
2	100	20 µg/ml	20 µg/ml	39.71 µg/ml	99.286 ± 0.00205
3	120	20 µg/ml	24 µg/ml	43.61 µg/ml	99.132 ± 0.00205
Formoterol Fumarate Dihydrate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Conc. Recovered	**%Recovery ± SD (n=3)**
1	80	0.6 µg/ml	0.48 µg/ml	1.05 µg/ml	100.222
2	100	0.6 µg/ml	0.6 µg/ml	1.16 µg/ml	99.323
3	120	0.6 µg/ml	0.72 µg/ml	1.29 µg/ml	99.932

Table 6: Accuracy of Dual Wavelength Method expressed in terms of % Recovery

Dual Wavelength Method					
Beclomethasone Dipropionate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Conc. Recovered	**%Recovery ± SD (n=3)**
1	80	20 µg/ml	16 µg/ml	35.83 µg/ml	99.541 ± 0.000471
2	100	20 µg/ml	20 µg/ml	39.76 µg/ml	99.416 ± 0.000471
3	120	20 µg/ml	24 µg/ml	43.73 µg/ml	99.393 ± 0.000471
Formoterol Fumarate Dihydrate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Conc. Recovered	**%Recovery ± SD (n=3)**
1	80	0.6 µg/ml	0.48 µg/ml	1.08 µg/ml	100.374 ± 0.000471
2	100	0.6 µg/ml	0.6 µg/ml	1.19 µg/ml	99.807 ± 0.000471
3	120	0.6 µg/ml	0.72 µg/ml	1.31 µg/ml	99.929 ± 0.000471

Table 7: Accuracy of RP-HPLC Method expressed in terms of % Recovery

RP - HPLC Method					
Beclomethasone Dipropionate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Total Conc.	**%Recovery ± SD (n=3)**
1	80	100 µg/ml	80 µg/ml	180 µg/ml	99.250 ± 15.735
2	100	100 µg/ml	100 µg/ml	200 µg/ml	100.591 ± 11.991
3	120	100 µg/ml	120 µg/ml	220 µg/ml	100.111 ± 3.237
Formoterol Fumarate Dihydrate					
Sr. No.	% Spiked	Conc. Of Test	Standard Conc. Added	Total Conc.	**%Recovery ± SD (n=3)**
1	80	3 µg/ml	2.4 µg/ml	5.4 µg/ml	100.979 ± 0.035
2	100	3 µg/ml	3 µg/ml	6 µg/ml	100.461 ± 1.215
3	120	3 µg/ml	3.6 µg/ml	6.6 µg/ml	104.306 ± 4.201

Robustness:

The robustness of the developed RP-HPLC method was determined by DOE Approach. Here the method was tested for robustness by deliberately changing the chromatographic conditions such as; Flow Rate, Percentage ACN and pH of the mobile phase. In a 3 level Factorial Design of response surface methodology the RP-HPLC method was analysed for robustness. The responses taken by changing the above chromatographic conditions were; Retention Time (min), Area (mV. s) and Asymmetry Factor.

32 trials were taken based on the factorial design created using Design Experts, out of which the 12th trial was the best trial having lowest asymmetry factor. This trail had a mobile phase pH of 4.5, a flow rate of 1ml/min and the percentage ACN was 70. It was also noted that the % RSD values of the retention times of both drugs did not exceed 2 hence proving that even with small, deliberate variation in the critical parameters the method is robust.

From the contour plots (**Figure 7 and 8**) made using Design Experts it may be concluded that with the increase in the flow rate and the %ACN there is an increase in the retention times as well as the area of the peaks of both the drugs. It may also be seen from **Figure 9** that despite the small changes in the chromatographic conditions the asymmetry factor is less than 2.

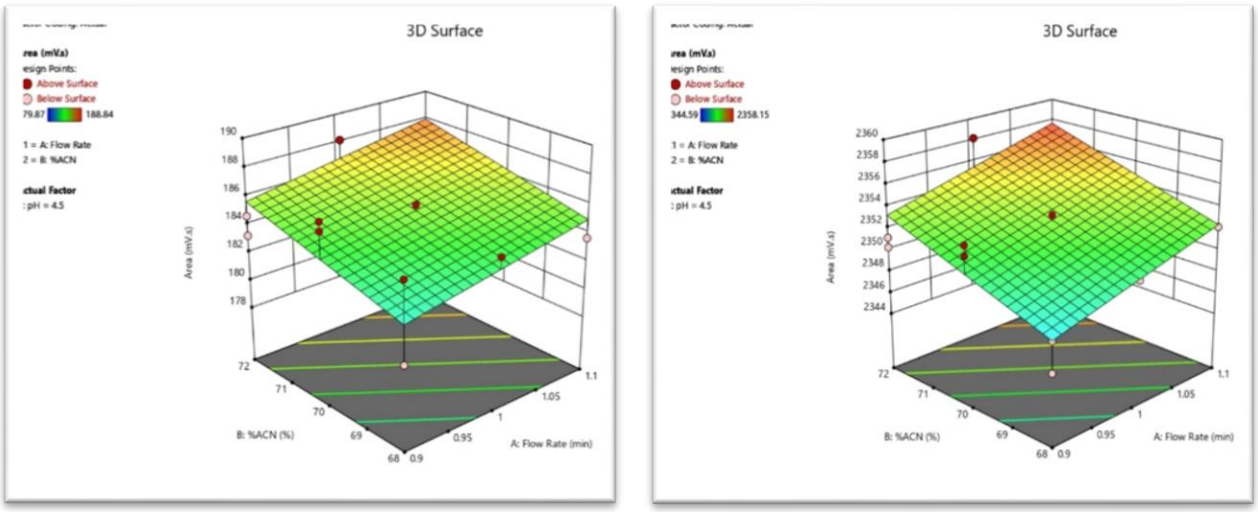

Figure 7: Contour plots of the areas of BDP (left) and FFD (right) with changes in flow rate and %ACN

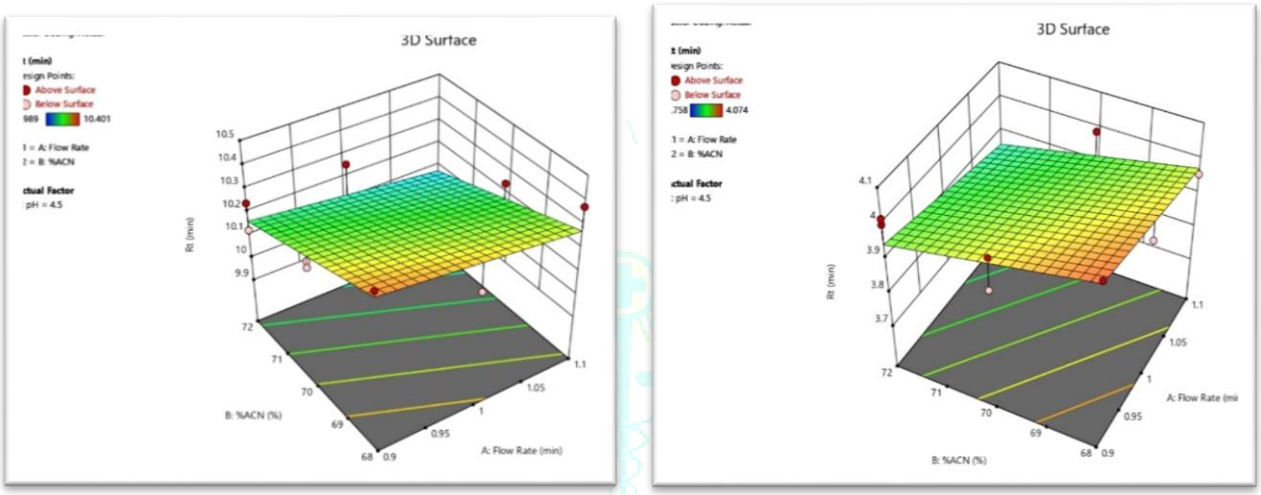

Figure 8: Contour plots of the retention times of BDP (left) and FFD (right) with changes in flow rate and %ACN

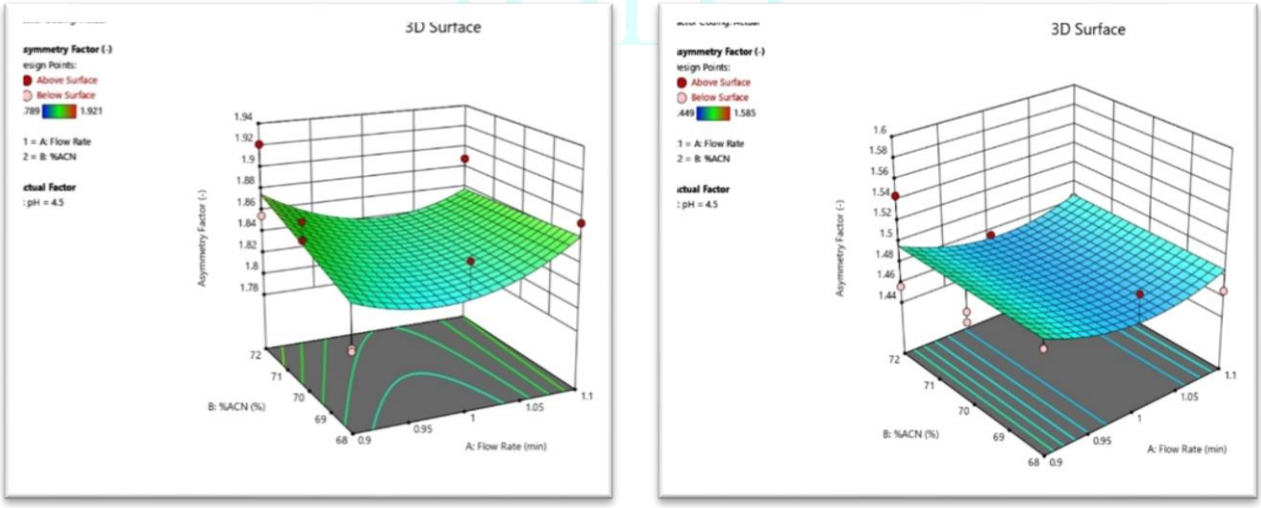

Figure 9: Contour plots of the asymmetry factors of BDP (left) and FFD (right) with changes in flow rate and %ACN

Specificity:

The specificity of the developed RP-HPLC method was done by injecting the following solutions into the HPLC system; Blank solution (Diluent used in the sample preparation), Standard drug solutions of both drugs (BDP and FFD) and Test solution (Formulation or Simulated mixture prepared in laboratory). It may be seen that there is no peaks in the blank solution as shown in **Figure 10** which depicts that the diluent used in the sample preparation does not produce any interference during test measurement. Also as the Standard drug solutions peaks (shown in **Figure 11**) resemble that of the Test solution (shown in **Figure 12**) it can be concluded that the method is specific.

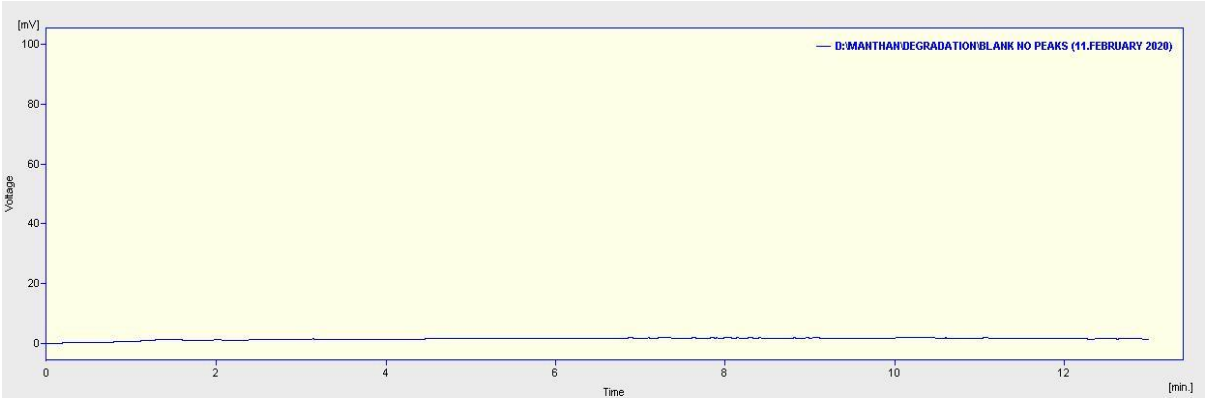

Figure 10: Chromatogram of the Blank solution (Diluent used in the sample preparation)

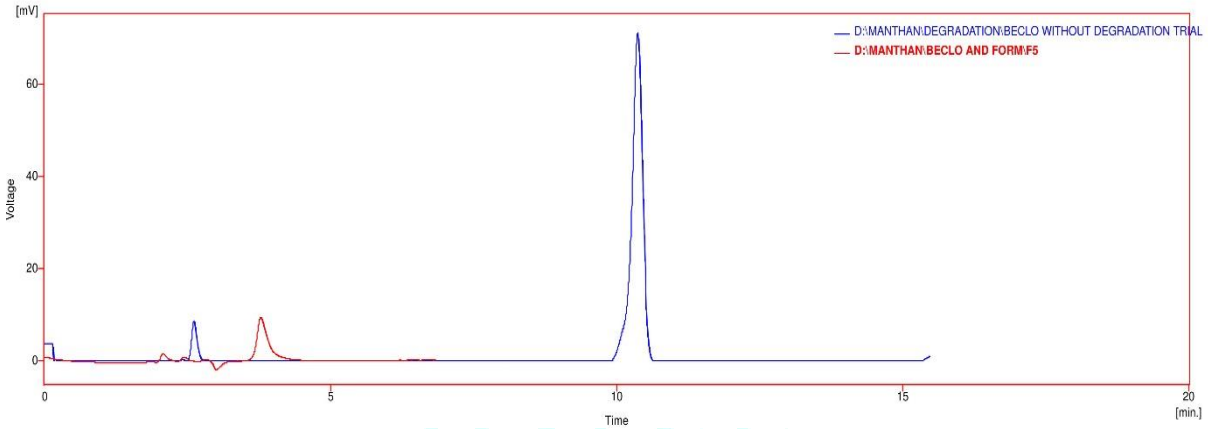

Figure 11: Chromatograms of the Standard drug solutions – API

(Blue – Beclomethasone Dipropionate, Red – Formoterol Fumarate Dihydrate)

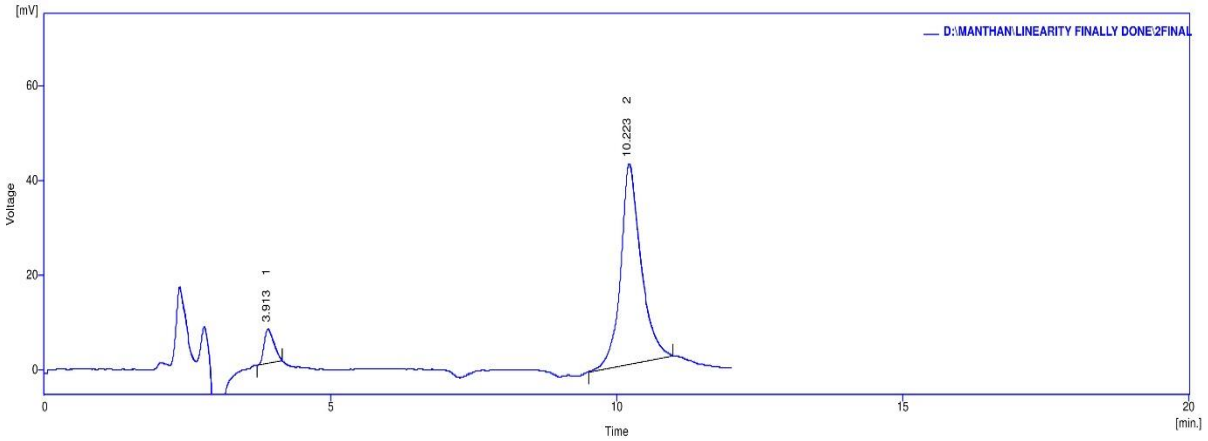

Figure 12: Chromatogram of the Test solution (Formulation)

System Suitability Testing for RP-HPLC:

The system suitability tests which were applied for the RP-HPLC Method were Retention Time, Theoretical Plates, Asymmetry Factor, Area of the Peaks and Resolution between the peaks as shown in **Table 8**. Retention times of BDP and FFD as well as Resolution between the peaks were having a %RSD value less than 2.

Table 8: System suitability testing data representing the Retention Times, Theoretical Plates, Asymmetry Factor, Area of Peaks and Resolution of BDP and FFD

Conc. (µg/ml)		Retention Time (min)		Theoretical Plates		Asymmetry Factor		Area of Peaks (mV. s)		Resolution
BDP	FFD	BDP	FFD	BDP	FFD	BDP	FFD	BDP	FFD	
50	1.5	10.177	3.913	46837	24348	1.38	1.73	437.2	35.3	13.452
100	3	10.223	3.913	48180	22698	1.42	1.70	1037.8	84.3	13.563
150	4.5	10.223	3.913	45517	20520	1.67	1.85	1673.7	135.3	13.296
200	6	10.190	3.907	47867	21138	1.78	1.44	2353.2	185.6	13.789
250	7.5	10.177	3.907	48673	19793	1.39	1.79	2945.7	230	13.772

Comparison of the Methods

Parameters	Drug	1st Derivative Method	Dual Wavelength Method
Limit of Detection	Beclomethasone Dipropionate	0.739 µg/ml	0.127 µg/ml
	Formoterol Fumarate Dihydrate	0.183 µg/ml	0.016 µg/ml
Limit of Quantification	Beclomethasone Dipropionate	2.24 µg/ml	0.384 µg/ml
	Formoterol Fumarate Dihydrate	0.556 µg/ml	0.049 µg/ml

CONCLUSION

A number of analytical methods are discussed in the given paper about the simultaneous estimation of Beclomethasone Dipropionate and Formoterol Fumarate Dihydrate in Bulk and in Pharmaceutical Formulations. Out of these methods, the Dual Wavelength Method is proposed to have a higher sensitivity as compared to the already existing methods to analyse this combination. A LC-MS compatible RP-HPLC method was also developed using 50mM Ammonium Acetate as the buffering agent in the aqueous part of the mobile phase, this method may be widely used for the estimation of this pharmaceutical combination.

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