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"RP-HPLC Method for Simultaneous Quantification of Vilanterol and Fluticasone Furoate: Development and Validation"

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Abstract
This study aimed to develop and validate an RP-HPLC method for quantifying Vilanterol and Fluticasone Furoate in pharmaceutical formulations used for respiratory disorders. The method utilized an Agilent Poroshell EC-120 C18 column with a mobile phase of 0.1% Perchloric acid and Acetonitrile (35:65). Key parameters like wavelength, column temperature, flow rate, and injection volume were optimized. Method validation followed ICH guidelines, ensuring specificity, linearity (R^2 = 1 over ranges of 4-6 µg/ml for Vilanterol and 16-24 µg/ml for Fluticasone Furoate), LOD (0.03 µg/ml for Vilanterol, 0.02 µg/ml for Fluticasone Furoate), LOQ (0.09 µg/ml for Vilanterol, 0.07 µg/ml for Fluticasone Furoate), precision, accuracy, and robustness. Results confirmed the method's efficiency and suitability for simultaneous drug determination in formulations.

Keywords: HPLC, Fluticasone, Vilanterol, Pharmaceutical, Spectrometer, Chromatography.

1. INTRODUCTION

RP-HPLC is the most popular mode of HPLC where the stationary phase is nonpolar, and the mobile phase is polar aqueous. This mode is the reverse of NP-HPLC in which the stationary phase is more polar than the mobile phase [34, 37]. Usually, RP-HPLC uses a stationary phase such as C18 silica with a moderately polar aqueous mobile phase. A popular RP-HPLC stationary phase is surface-modified silica, RMe2SiCl, where R is an unbranched alkyl group like C18H37 or C8H17. These silica-based reversed-phase sorbents are also referred to as “bonded-phase” materials. The eluent in RP-HPLC is usually a solution of water and a miscible organic solvent such as acetonitrile (ACN), methanol (MeOH), or tetrahydrofuran (THF) [37].
RP-HPLC is useful in the separation of many solutes of different functional groups. The chromatographic stationary phase is also an important factor that determines the separation result. Most RP-HPLC separations are carried out on C18 bonded phases with water and one or more organic solvents soluble in water [34-36]. For instance, the isolation of chlorophylls and their derivatives mainly employs reversed-phase stationary phases. In general, gradient elution is necessary for the proper separation of carotenoids. Because of the differences in the wavelengths of maximum absorbance of various pigment fractions and the difficulties in their identification, diode array detection (DAD) or mass detection (MS) has been widely studied. DAD has been applied for the quantitative analysis at the maximum absorbance of each fraction of chili color pigments [35].

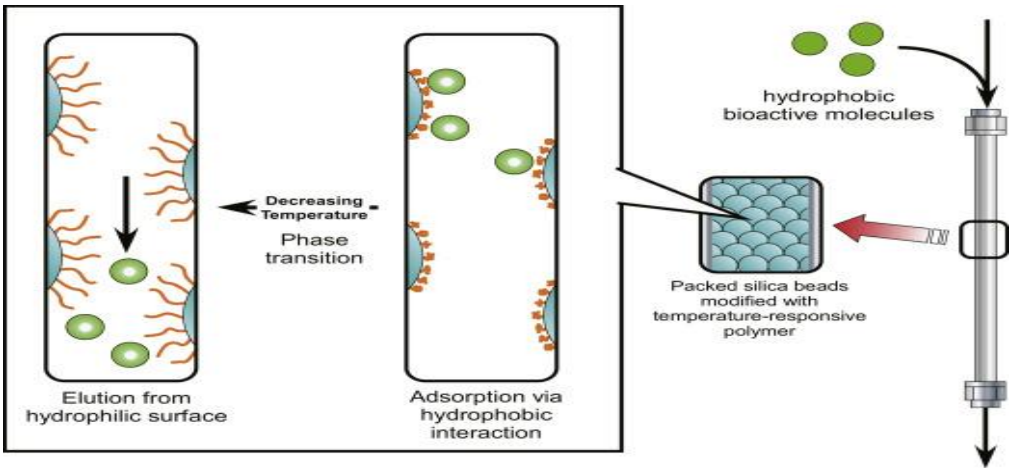
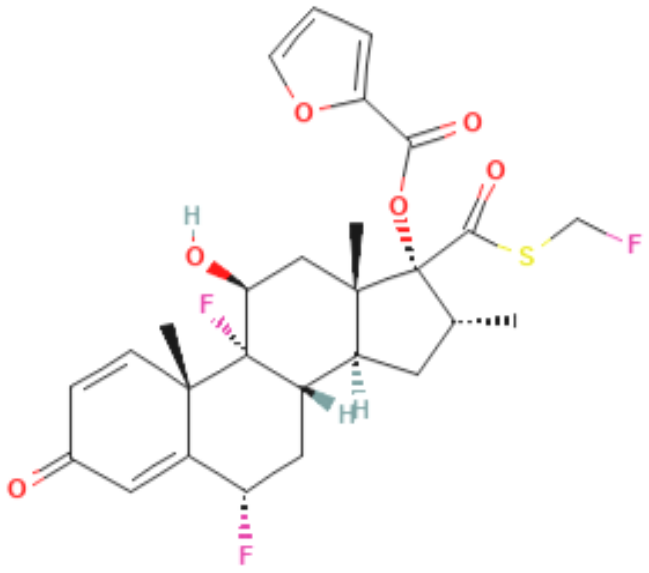


Figure 1: Reverse Phase Chromatography

Vilanterol and Fluticasone Furoate are co-formulated in inhalers that are widely used in the management of respiratory disorders including asthma and COPD. Vilanterol is a long-acting β2 agonist (LABA) that helps in the relaxation of the muscles around the airways to enhance bronchodilation. On the other hand, Fluticasone Furoate is a corticosteroid that has a strong anti-inflammatory action due to its ability to decrease inflammation and swelling in the airways [33, 36]. Vilanterol and Fluticasone Furoate in a single inhaler offer a dual mechanism of action that relieves bronchoconstriction and inflammation that is characteristic of asthma and COPD. This co-ordinate action does not only help in better symptom management but also in the improvement of lung function and decrease in the number of exacerbations thus greatly improving the quality of life of patients [33, 36].
Vilanterol and Fluticasone Furoate, co-formulated in inhalers for respiratory disorders like asthma and COPD, combine a long-acting β2 agonist (Vilanterol) for bronchodilation and a corticosteroid (Fluticasone Furoate) for anti-inflammatory action, improving symptom management and lung function. This study focuses on developing an RP-HPLC method to quantify these drugs in pharmaceutical formulations. Critical steps include selecting chromatographic conditions (e.g., C18 column, mobile phase of water and acetonitrile), optimizing gradient elution parameters for optimal separation. It is therefore important to validate the RP-HPLC method according to the ICH guidelines to determine its reliability and accuracy. Specificity tests confirmed its ability to accurately quantify Vilanterol and Fluticasone Furoate amidst formulation components. Linearity was established across specified concentration ranges, ensuring direct proportionality between analyte concentration and peak area. Accuracy assessments verified the method's reliability in measuring true standard values. Precision studies demonstrated consistent results under varied conditions. Limits of detection and quantification were determined to ensure sensitivity. Robustness testing validated the method's reliability under minor parameter variations.

2. BACKGROUND

Drug Profile 1: *Fluticasone Furoate* [8-15]
Fluticasone furoate, available under trade names like Veramyst and Arnuity Ellipta, is a synthetic trifluorinated corticosteroid recognized for its potent anti-inflammatory properties, particularly effective in managing asthma and allergic conditions. Administered via inhalation or nasal spray, it acts by binding strongly to glucocorticoid receptors, thereby inhibiting various pro-inflammatory factors involved in the pathogenesis of asthma. Despite its rapid absorption with peak plasma concentrations reached within 0.5-to-1-hour post-inhalation, fluticasone furoate exhibits low systemic bioavailability due to extensive first-pass metabolism via hepatic CYP3A4 enzymes.

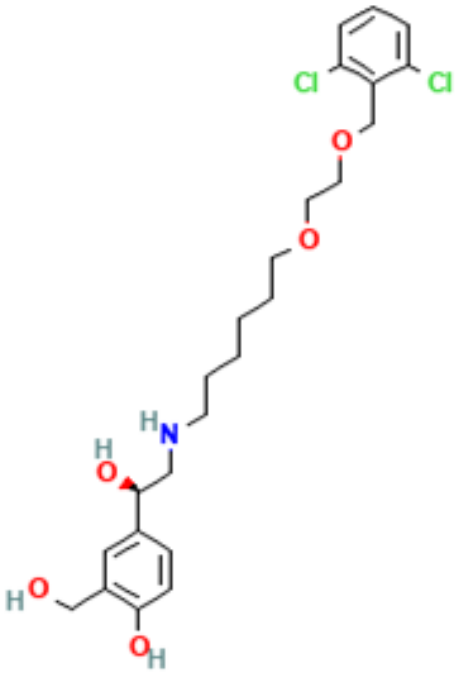
Drug Name	**Fluticasone Furoate**
IUPAC Name	"[(6*S*,8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-6,9-difluoro-17-(fluoromethylsulfanylcarbonyl)-11-hydroxy-10,13,16-trimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] furan-2-carboxylate"
Molecular Formula	$C_{27}H_{29}F_3O_6S$
Molecular weight	538.6 g/mol
Structure	
CAS No.	397864-44-7

The clinical efficacy of fluticasone furoate in asthma treatment is characterized by a delayed onset of action, often requiring 1 to 2 weeks or longer for maximum symptom relief. This delayed response is attributed to its high local anti-inflammatory activity at the site of action in the lungs, minimal systemic absorption (approximately 1.3%), and negligible pharmacological activity of its metabolites. In studies involving subjects with asthma, the drug's impact on cortisol levels in serum and urine was minimal even at high doses, demonstrating its favorable safety profile compared to systemic corticosteroids.

Safety considerations include its predominantly hepatic metabolism, where it is primarily metabolized to inactive forms before excretion mainly through feces. Fluticasone furoate is highly protein-bound (>99%) in serum, primarily to albumin and α1-acid glycoprotein, further limiting its systemic distribution. While it has shown no evidence of carcinogenicity or mutagenicity in studies, caution is advised regarding potential adrenal suppression, especially in vulnerable populations such as those with hepatic impairment.

Drug Profile 2: Vilanterol [16-22]

Vilanterol is a selective long-acting β2-adrenergic agonist (LABA) used for treating COPD and asthma. It offers 24-hour activity, addressing issues of patient compliance with its once-daily dosing regimen. Structurally derived from salmeterol, vilanterol is highly selective for β2-adrenoceptors and demonstrates faster onset and longer duration of action compared to salmeterol. It functions by stimulating adenylyl cyclase, leading to increased cyclic AMP levels, which relax bronchial smooth muscle and inhibit hypersensitivity mediator release from lung mast cells.

Drug Name	**Vilanterol**
IUPAC Name	"4-[(1*R*)-2-[6-[2-[(2,6-dichlorophenyl) methoxy] ethoxy] hexylamino]-1-hydroxyethyl]-2-(hydroxymethyl)phenol"
Molecular Formula	$C_{24}H_{33}Cl_2NO_5$
Molecular weight	486.4 g/mol
Structure	
CAS No.	503068-34-6

Approved formulations include combinations with fluticasone furoate (BREO ELLIPTA), umeclidinium bromide (ANORO ELLIPTA), and both (TRELEGY ELLIPTA). It achieves peak plasma levels within 5 to 15 minutes post-inhalation, with steady state reached after 14 days of repeat dosing. Metabolized primarily by CYP3A4, vilanterol is excreted mostly via urine and feces, with an effective half-life of approximately 11 hours. Adverse effects include those typical of excessive β-adrenergic stimulation, with precautionary notes on potential systemic and developmental impacts observed in animal studies.

3. AIM AND OBJECTIVE

Aim: The aim of this research is to establish a sensitive, accurate and precise Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the determination of Vilanterol and Fluticasone Furoate in bulk and in pharmaceutical dosage forms.

Objectives:

I.Preliminary Analysis of the Drugs: Conduct initial assessments and characterizations of Vilanterol and Fluticasone Furoate to understand their chemical properties and behaviors.

II.Development of the RP-HPLC Method: Create an optimized RP-HPLC method that can accurately and efficiently separate and quantify Vilanterol and Fluticasone Furoate simultaneously.

III.Validation of the Developed Method: Validate the RP-HPLC method according to the International Council for Harmonisation (ICH) Q2 (R1) guidelines to ensure its reliability, accuracy, precision, specificity, linearity, range, and robustness.

4. MATERIAL AND METHOD

Materials required

- *Drug*

Fluticasone Furoate, Vilanterol [Adhar Life Sciences Pvt. Ltd., Solapur, India]

- *Chemicals*

Perchloric Acid [Molychem, Mumbai]
Water [Lab Q ultra-Water System]
Acetonitrile [Qualigens, Mumbai]

Instruments used

HPLC	Agilent 1260 Infinity II
Software	Open Lab ezchrom version A.04.08
Channel vacuum Degasser & Mixer	Infinity Standard Degasser G1322A
Gradient Pump	Quaternary Pump G1311B
Injector	Auto injector G7129A
UV-Detector	Diode Array Detector- G4212B (DAD)
Column	Agilent Poroshell EC-120 C18
Analytical Balance	Aczet CY224C
Nylon 6,6 membrane 0. 45µm 47mm Filters	Pall pvt. Ltd
All Glass Filter Holder- 47mm (1L flask, 300ml funnel)	Borosil Glass works Ltd., Mumbai
Melting Point Apparatus	Veego
RC membrane 0.45µm 15mm Syringe Filters	AxivaSichem Biotech
Ultra Sonicator/ water bath	Labman

Methods

➢ ***Preliminary Analysis of Drug: Fluticasone Furoate and Vilanterol***

The sample of Fluticasone Furoate was first observed for its colour and texture and then it was taken in test tubes and observed for solubility in water, Acetonitrile, and methanol. Its melting point was determined using a capillary tube in a melting point apparatus.

The sample of Vilanterol was observed for its color and texture. The sample of Vilanterol was taken in test tubes and observed for solubility in water, acetonitrile, and methanol. The sample of Vilanterol was taken in capillary tube and kept in melting point apparatus.

➢ **HPLC Method Development**

- **Chromatographic Conditions**

a. Oven Temp: 30°C
b. Flow rate: 0.8 ml/min.
c. Mobile Phase- 0.1% Perchloric acid: Acetonitrile (35: 65%, v/v)
d. Runtime: 10 minutes
e. Injection Volume: 10µl
f. Wavelength: 250 nm
g. Diluent: 0.1% Perchloric acid: Acetonitrile (50: 50%, v/v)
h. Column: Agilent Poroshell EC 120 C18 (150 x 4.6 mm, 4 µ)

- **Standard Preparation**

To prepare the *Fluticasone Furoate Standard Stock Solution-I (*FSSS-I), dissolve 10 mg of Fluticasone Furoate in a 10 ml volumetric flask, add 5 ml of diluent, mix for 2 minutes, and dilute to 10 ml with diluent to achieve a concentration of 1000 µg/ml. For the *Vilanterol Standard Stock Solution-I* (VSSS-I), dissolve 5 mg of Vilanterol in a 10 ml volumetric flask, add 5 ml of diluent, mix for 2 minutes, and dilute to 10 ml with diluent for a concentration of 500 µg/ml. Combine 2.0 ml of FSSS-I and 1.0 ml of VSSS-I in a 100 ml volumetric flask, add 50 ml of diluent, vortex, and dilute to 100 ml with diluent to obtain final concentrations of 20 µg/ml Fluticasone Furoate and 5 µg/ml Vilanterol.

- **Drug Product Sample Preparation for Assay**

The contents of ten capsules were weighed to calculate the average weight of one capsule. A powder weight equivalent to 200 µg of Fluticasone Furoate and 50 µg of Vilanterol was weighed into a 10 ml volumetric flask. 5 ml of diluent was added, sonicated for 5 minutes, and the volume was made up to 10 ml with diluent to achieve final concentrations of 20 µg/ml Fluticasone Furoate and 5 µg/ml Vilanterol.

- **Selection of Wavelength**

The sample was scanned from 200-400 nm with DAD detector. The Wavelength selected for analysis chosen was 250 nm on basis of appropriate intensity of Fluticasone &Vilanterol.

- **Method Validation**

- **Specificity & Assay**

Individual samples of Fluticasone Furoate of 20 µg/ml and Vilanterol of 5 µg/ml were prepared and peaks were for identified from Retention Time. Blank was injected to ensure there is no blank peak interfering with the main analyte peaks. Assay was calculated by using following formula:

$$\% \text{ Assay} = \frac{\text{Sample Area}}{\text{Standard Area}} \text{ x } 100$$

- **Repeatability& System Suitability**

A single sample was prepared, from which six injections were made and checked for system suitability, evaluating parameters such as “retention time”, “theoretical plates”, “asymmetry” (tailing factor), and “resolution”.

- **Linearity & Range**

Five samples of varying concentrations, ranging from 80% to 120%, were prepared as follows:

% Level	Fluticasone Furoate Conc. (µg/ml)	Vilanterol Conc. (µg/ml)
80	16	4
90	18	4.5
100	20	5
110	22	5.5
120	24	6

For sample preparations, X ml of Fluticasone Furoate (FSSS-I) and Y ml of Vilanterol (VSSS-I) were added to 100 ml diluent to achieve the specified concentrations:

X ml of FSSS-I	Y ml of VSSS-I	Diluted to
1.6	0.8	100 ml
1.8	0.9	100 ml
2.0	1.0	100 ml
2.2	1.1	100 ml
2.4	1.2	100

- **Accuracy**

Samples spiked at 80%, 100%, and 120% concentrations for Fluticasone Furoate and Vilanterol were analyzed in triplicate to determine %RSD and %recovery.

- **LOD/ LOQ**

LOD/ LOQ **w**as calculated for both drugs by using ANOVA technique.

Formula:

$$\text{LOD} = \frac{3.3 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

$$\text{LOQ} = \frac{10 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

- **Robustness**

The robustness of the method was determined by ± 2˚C change in column temperature and ± 20% change in the concentration of perchloric acid and the % Assay was determined for each of the changes.

Condition	Increased	Normal	Decreased
Column Oven Temperature	32˚C	30˚C	28˚C
Perchloric acid Concentration	0.12%	0.1%	0.08%

- **Intra & Inter-day Precision**

Intra-day and inter-day precision of a single mixture of working standards and drug product samples were evaluated by injecting it twice on the same day and on a subsequent day, respectively. % Assay and % RSD were calculated for each interval, while solution stability was also assessed.

6. RESULT AND DISCUSSION

6.1 Preliminary Analysis of Drug

Table 2: Preliminary Data of Drugs

Sr. No.	Properties	Fluticasone Furoate	Vilanterol
1.	**Colour**	White to off white Powder	Light yellow to yellowish in colour
2.	**Solubility**	Fluticasone Furoate is insoluble in water, and slightly soluble in acetone, dimethyl sulfoxide and ethanol.	Vilanterol is insoluble in water and slightly soluble in methanol, ethanol, acetonitrile and propan-2-ol.
3.	**Melting Point**	The melting of Fluticasone Furoate was found to be 251°C.	The melting of Vilanterol was found to be 132°C.

Selection of Wavelength

For wavelength selection, UV spectrum was obtained in Diluent (0.1% Perchloric acid: ACN (50:50). The sample was scanned from 190-400 nm with PDA detector. The Wavelength selected for analysis was 250 nm based on appropriate intensity of Vilanterol & Fluticasone Furoate.

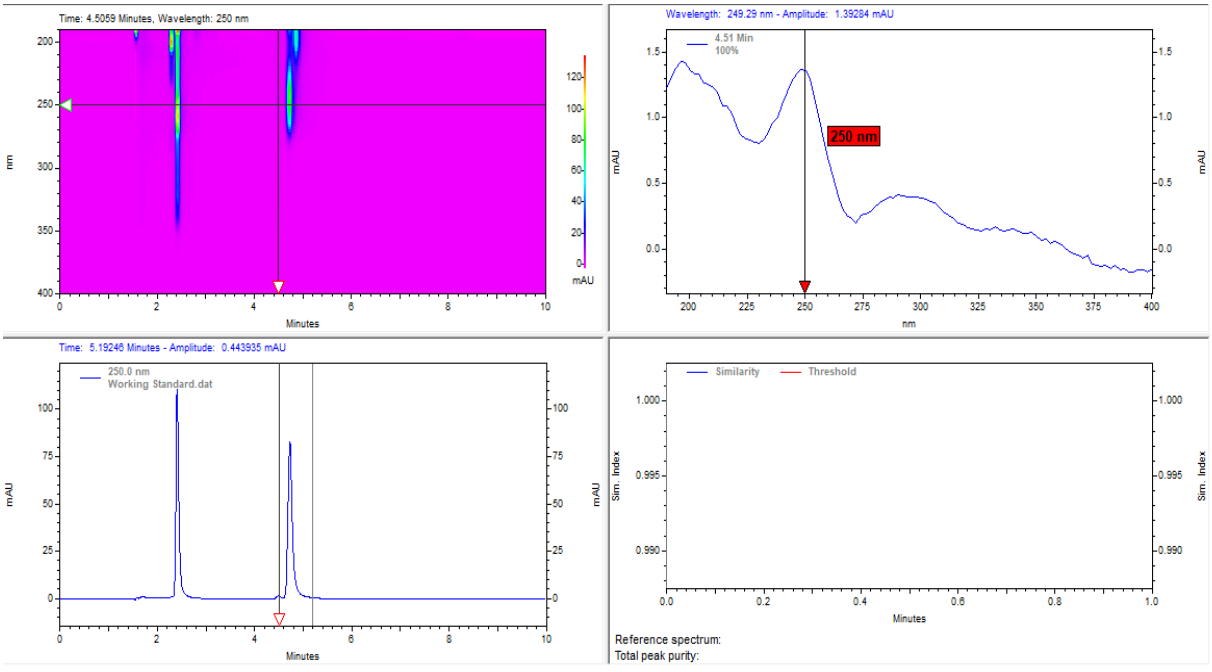


Figure 2: Wavelength Selection of Fluticasone Furoate & Vilanterol

6.2 HPLC Method for Fluticasone Furoate and Vilanterol

7.2.1. Method Development HPLC

A few important selections were made before initiating the development work. Stationary Phase was selected based on the polarity. Based on literature review, the mobile phase of 0.1% 0.1% Perchloric Acid and Acetonitrile was chosen for the HPLC analysis of Fluticasone Furoate & Vilanterol with Agilent Poroshell EC-120 C18 column with dimension 150 x 4.6 mm, 4-micron particle size. Column temperature at 30°C and injection volume at 10 µl. Diluent as 50:50 0.1% Perchloric Acid and Acetonitrile. The trials for method development are mentioned in table 3.

Table 3: Method Development for Fluticasone Furoate & Vilanterol HPLC

Trial No.	Mobile Phase	Ratio	Diluent	Flow Rate (ml/min)	Column (Dimension)	Wavelength
1	0.1% Perchloric Acid-ACN	50-50	50 0.1% Perchloric Acid-50 ACN	1	Agilent Poroshell EC120 C18 (150 x 4.6 mm, 4µ)	250 nm
2	0.1% Perchloric Acid-ACN	40-60	50 0.1% Perchloric Acid-50 ACN	1	Agilent Poroshell EC120 C18 (150 x 4.6 mm, 4µ)	250 nm
3	0.1% Perchloric Acid-ACN	35-65	50 0.1% Perchloric Acid-50 ACN	1	Agilent Poroshell EC120 C18 (150 x 4.6 mm, 4µ)	250 nm
4	0.1% Perchloric Acid-ACN	35-65	50 0.1% Perchloric Acid-50 ACN	0.8	Agilent Poroshell EC120 C18 (150 x 4.6 mm, 4µ)	250 nm

HPLC Results of Method Development

In method development across four trials, adjustments were made to optimize the chromatographic separation of Vilanterol and Fluticasone Furoate. In Trial 1, with a runtime of 20 minutes, Vilanterol eluted at 2.89 minutes and Fluticasone Furoate at 14.11 minutes. The concentration of 0.1% Perchloric acid was reduced by 10%, resulting in Trial 2 where Vilanterol eluted earlier at 2.08 minutes and Fluticasone Furoate at 4.95 minutes, achieving a resolution of 21.39 between their peaks.

In Trial 3, further adjustments aimed to improve peak shape by reducing the Perchloric acid concentration by 5%. This led to Vilanterol eluting at 1.92 minutes and Fluticasone Furoate at 3.75 minutes, with a resolution of 15.99. Additionally, the flow rate was decreased to 0.8 ml/min to enhance resolution and elution efficiency. Finally, in Trial 4, the flow rate of 0.8 ml/min maintained theoretical plates above 8000, asymmetry less than 2.0, and a resolution of 17.47 between Vilanterol and Fluticasone Furoate, with their respective retention times adjusted to 2.40 minutes and 4.69 minutes. These method refinements ensured robust chromatographic performance for accurate and efficient analysis of both compounds.

Table 4: Method development results of Fluticasone Furoate & Vilanterol

Trial No.	Vilanterol				Fluticasone Furoate			
	RT	Theoretical Plates	Asymmetry	Resolution	RT	Theoretical Plates	Asymmetry	Resolution
1.	2.89	9501	1.45	0.00	14.11	13618	1.30	37.28
2.	2.08	7322	1.35	0.00	4.95	13358	1.33	21.39
3.	1.92	6701	1.43	0.00	3.75	12399	1.40	15.99
4.	2.40	8400	1.51	0.00	4.69	14155	1.42	17.47

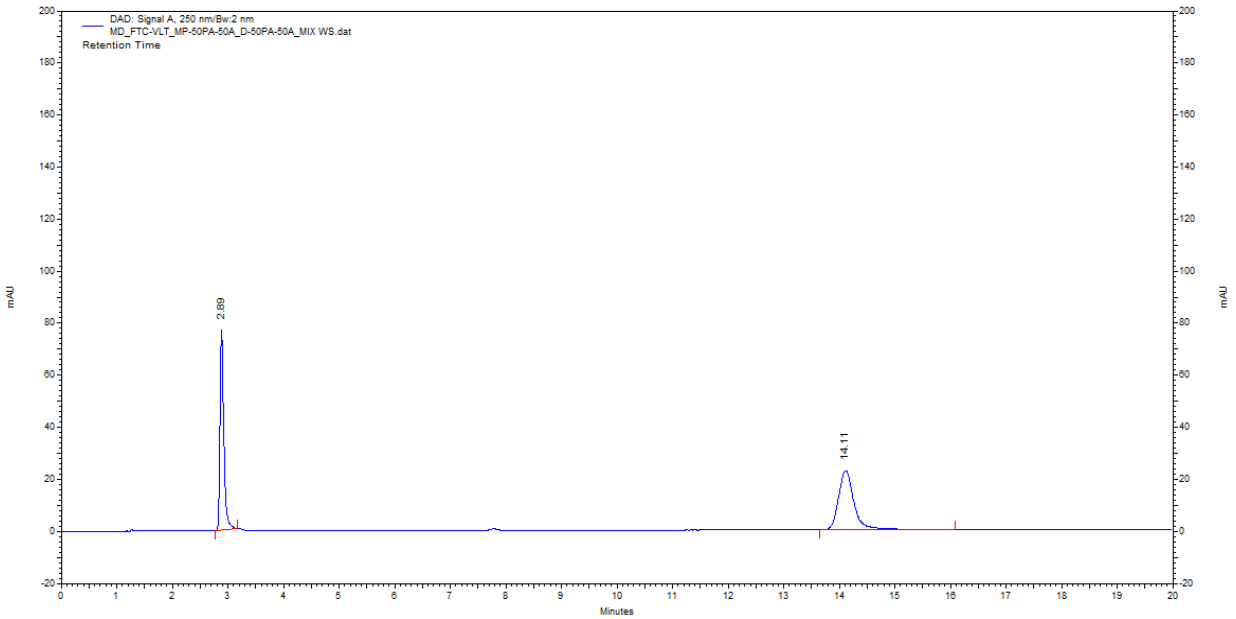


Figure 3: Method Development- "Trial 1"

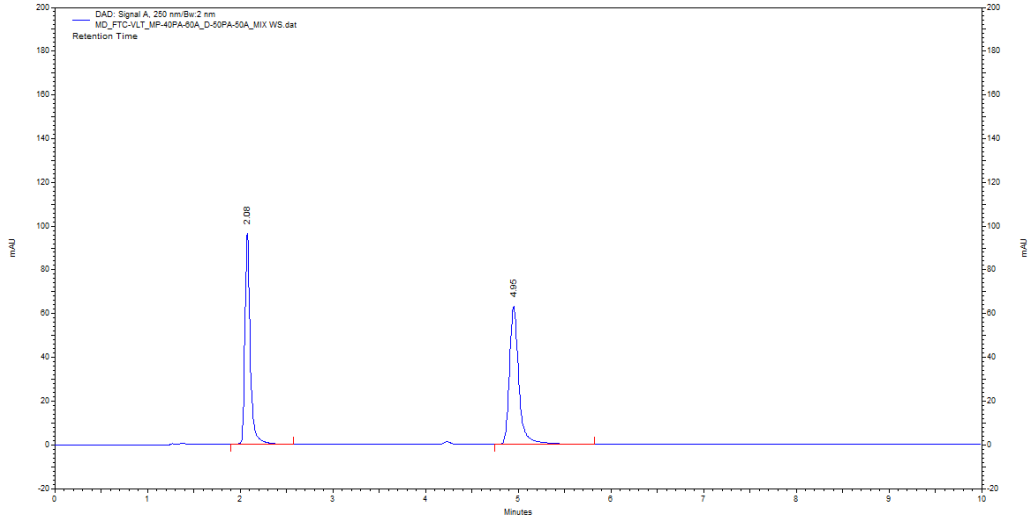


Figure 4: Method Development- "Trial 2"

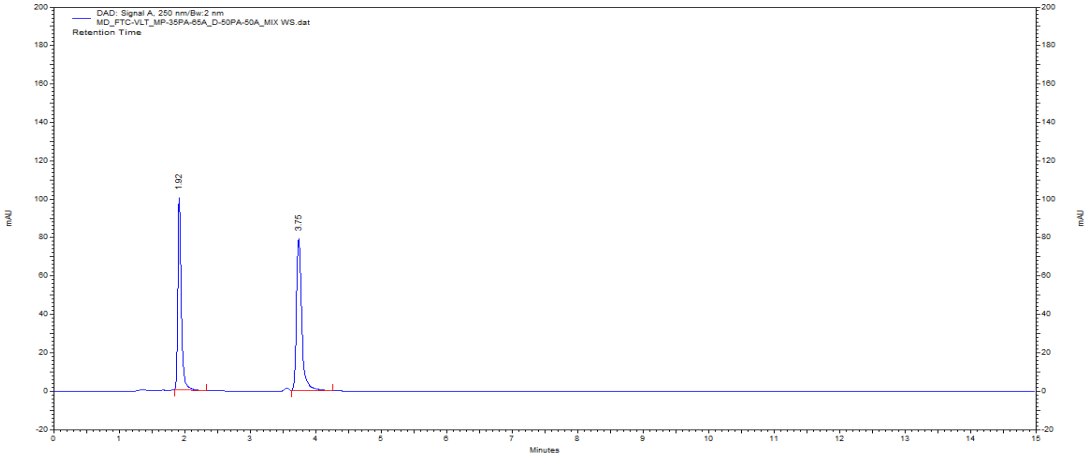


Figure 5: Method Development- "Trial 3"

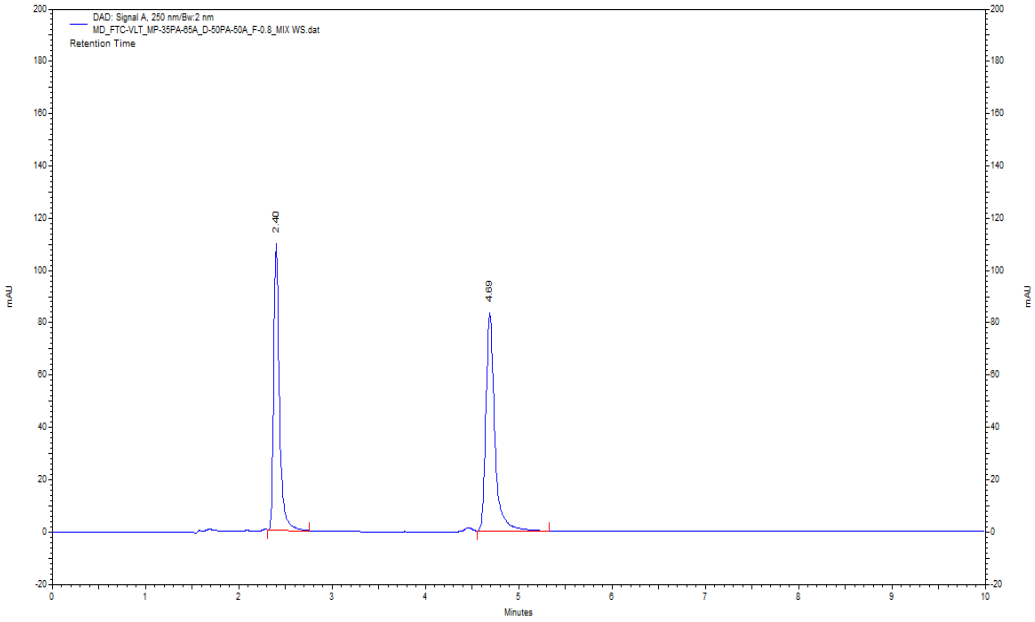


Figure 6: Method Development- "Trial 4"

Trial 4 was selected as the final method. The developed method can be used for individual estimation of Vilanterol and Fluticasone Furoate within 10 minutes.

Method Validation

I.Specificity: Specificity was performed to check if there was any interaction between the peaks from blank or the APIs. All the peaks were well separated. There were no interfering peaks in the chromatogram of the blank, at

retention time corresponding to the peak of Vilanterol and Fluticasone Furoate. The Retention time of Vilanterol and Fluticasone Furoate were found to be 2.41 minutes and 4.73 minutes, respectively. The % assay for Vilanterol and Fluticasone Furoate were found to be 99.78% and 99.74%, respectively.

Table 6: Specificity results of Fluticasone Furoate and Vilanterol

Sample ID	Vilanterol			Fluticasone Furoate		
	RT	Area	%Assay	RT	Area	%Assay
Blank	-	-	-	-	-	-
Vilanterol WS	2.41	962545	-	-	-	-
Fluticasone WS	-	-	-	4.73	1149652	
Mix WS	2.41	974715	-	4.73	1150809	-
Drug Product	2.41	972533	99.78	4.73	1147854	99.74

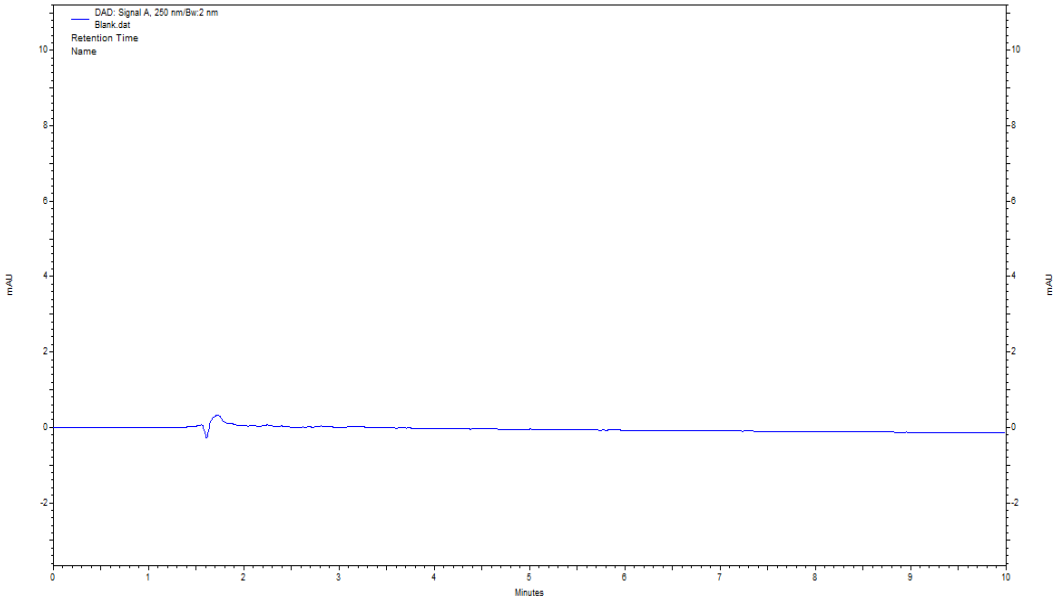


Figure 7: Specificity Blank-Diluent Chromatogram

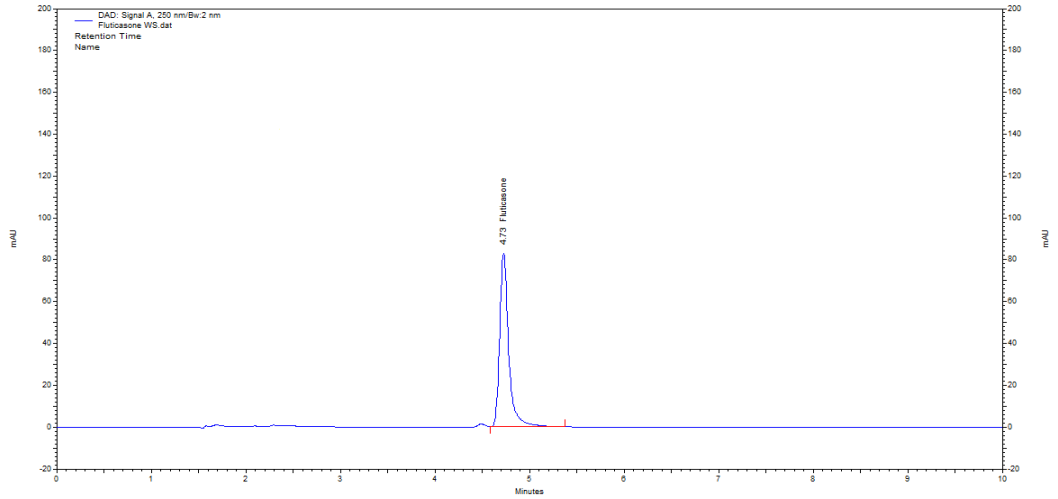


Figure 8: Fluticasone Furoate Working Standard

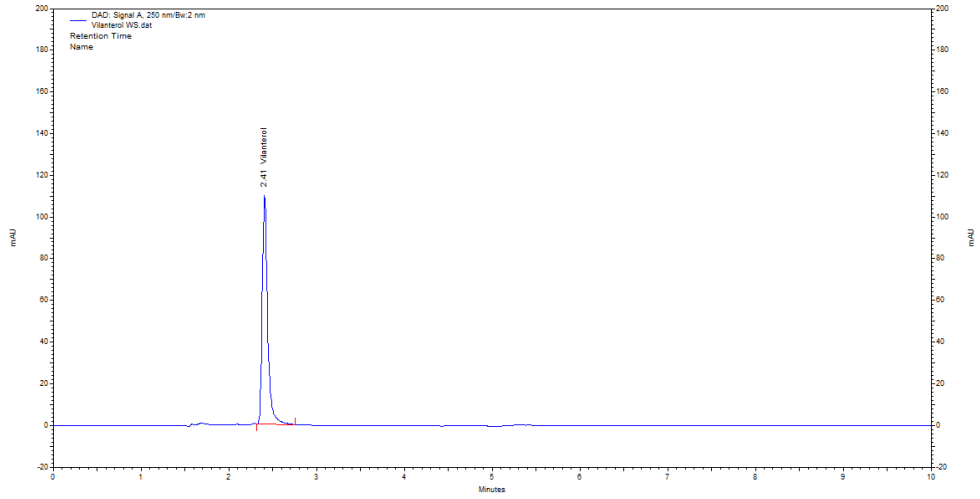


Figure 9: Vilanterol Working Standard

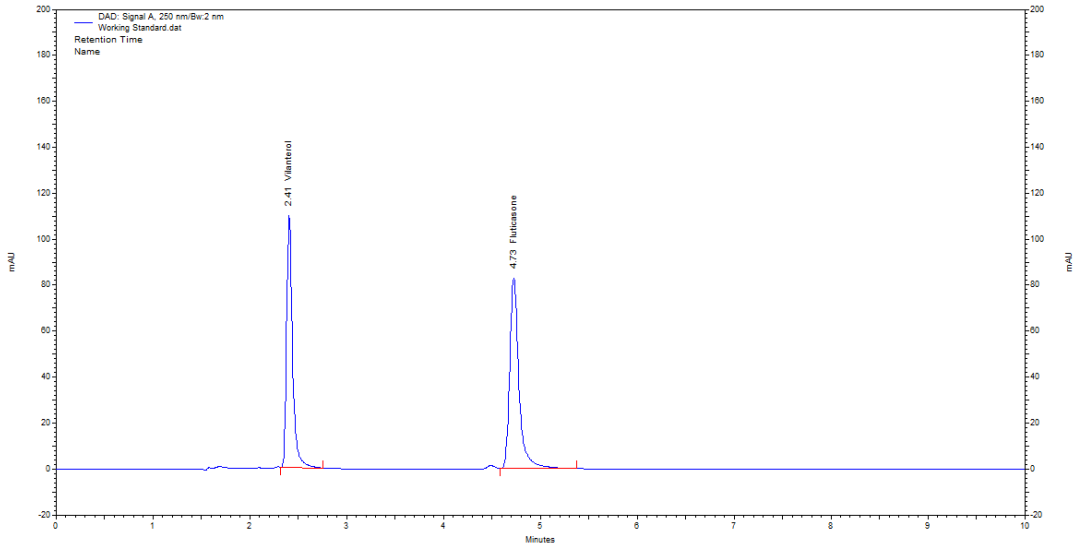


Figure 10: Mixture Working Standard

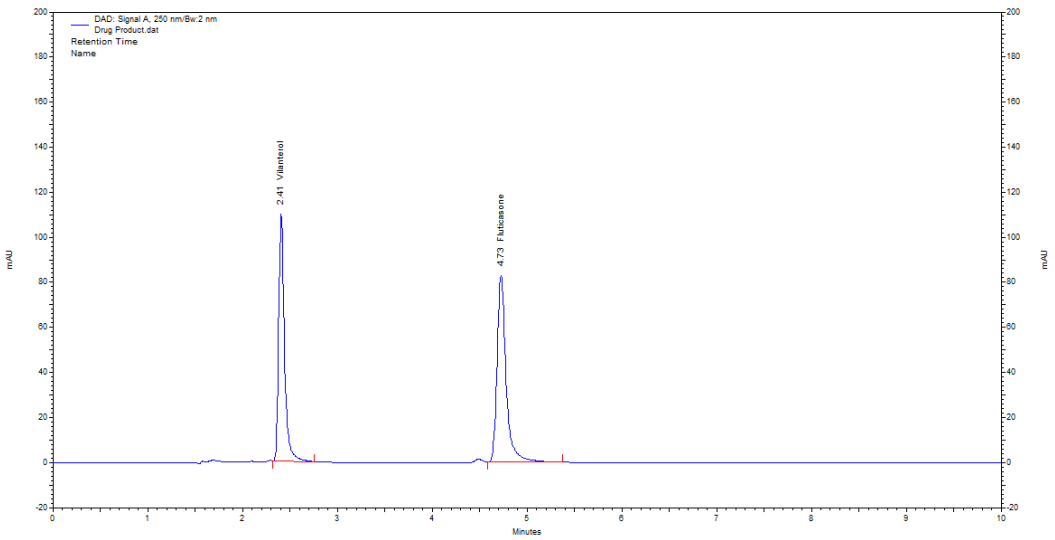


Figure 11: Drug Product

II.Instrument Precision and System suitability

Instrument precision was performed for both APIs. The reported peak area is shown below:

Table 7: Instrument precision of Vilanterol and Fluticasone Furoate

Sample ID	Vilanterol	Fluticasone
	Area	Area
Rep 1	974715	1150809
Rep 2	972287	1149125
Rep 3	973614	1155485
Rep 4	975545	1148965
Rep 5	971978	1145565
Rep 6	976524	1151454
Average	974110.5	1150233.83
STDEV	1809.276844	3287.30372
%RSD	0.19	0.29

From the above data, the %RSD for 6 replicate injections of Vilanterol and Fluticasone Furoate are 0.19% and 0.29% respectively. The %RSD is less than 2% as per the specification and guidance in ICH. The Chromatograms are given below:

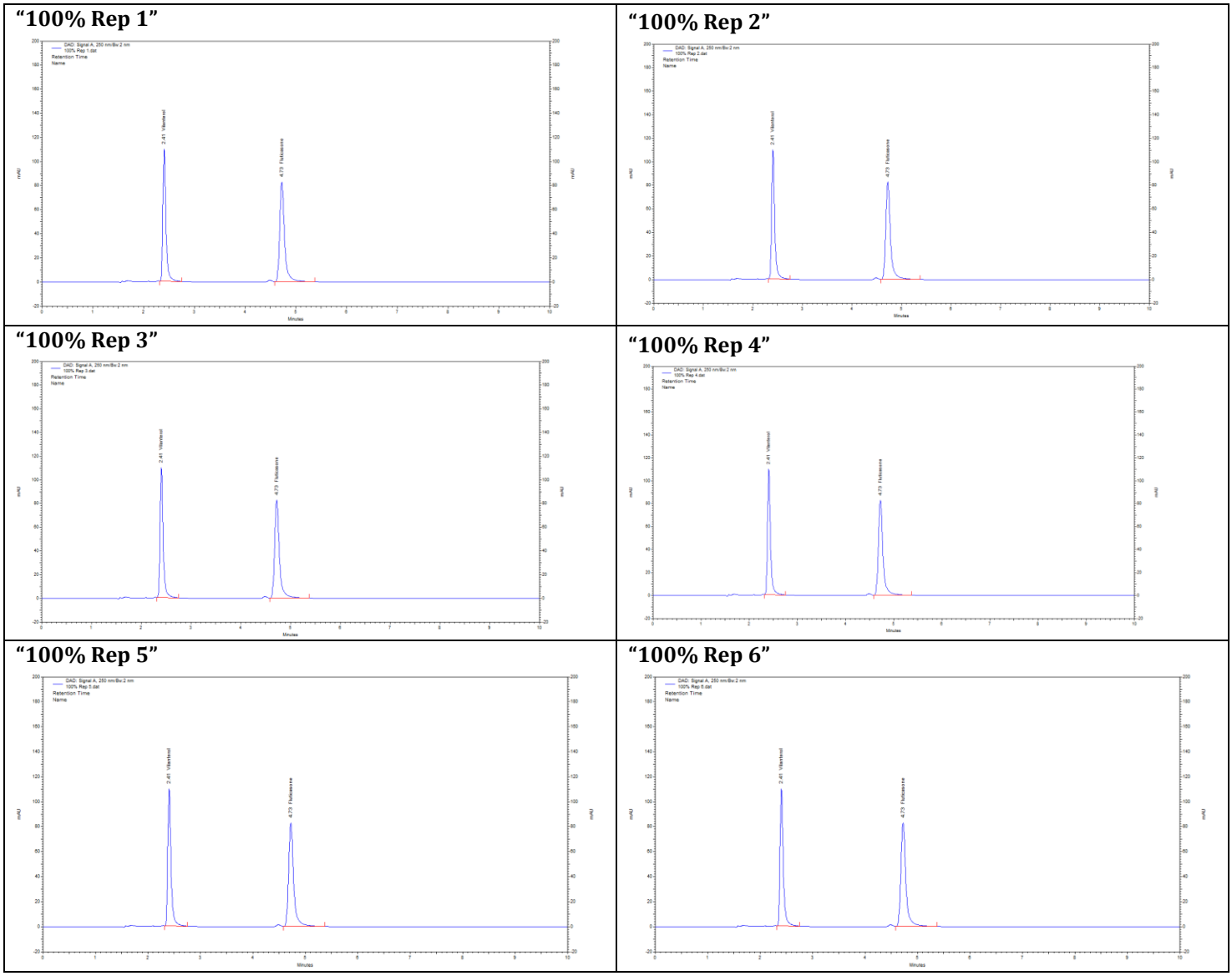


Figure 12: Instrument Precision of Vilanterol and Fluticasone Furoate

The system suitability criteria are per guidance ICH.

Criteria:
Theoretical Plates: more than 2000
Asymmetry: less than 2.0
Resolution: more than 2
%RSD of 6 replicates of working standard Retention time of main peak: less than 2%

Table 8: System suitability for Vilanterol

Vilanterol				
Sample ID	**RT**	**TP**	**Asymmetry**	**Resolution**
Rep 1	2.41	8428	1.49	0.00
Rep 2	2.41	8265	1.42	0.00
Rep 3	2.41	8147	1.39	0.00
Rep 4	2.41	8655	1.53	0.00
Rep 5	2.41	8351	1.51	0.00
Rep 6	2.41	8254	1.38	0.00
Average	2.41			
STDEV	0			
%RSD	0.00			

Table 9: System suitability for Fluticasone Furoate

Fluticasone Furoate				
Sample ID	**RT**	**TP**	**Asymmetry**	**Resolution**
Rep 1	4.73	13941	1.44	17.51
Rep 2	4.73	12598	1.38	17.51
Rep 3	4.73	13547	1.49	17.51
Rep 4	4.73	13222	1.52	17.51
Rep 5	4.73	13138	1.49	17.51
Rep 6	4.73	13788	1.42	17.51
Average	4.73			
STDEV	0			
%RSD	0.00			

Based on the limits mentioned, the equipment was found to be suitable for continuing the validations as the theoretical plates count was above 8000, the asymmetry was less than 2.

III.Linearity
Based on series of dilutions, linearity was performed. The Criterion for linearity is as below:
R^2 – more than 0.998
Linearity data for Vilanterol and Fluticasone Furoate is as below:

Table 10: Linearity data for Vilanterol

Vilanterol		
% Level	**Conc (ug/ml)**	**Area**
80	4	781421
90	4.5	878402
100	5	974715
110	5.5	1069844
120	6	1166756

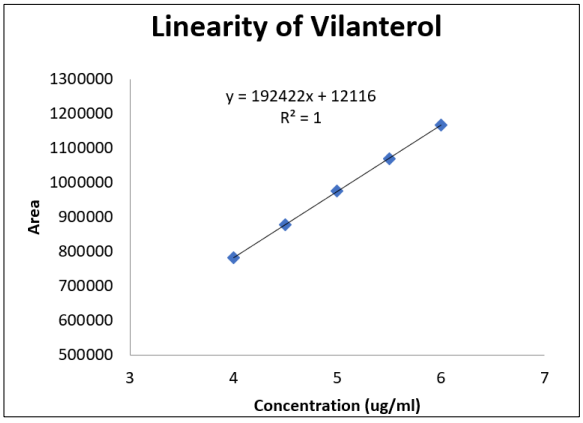


Figure 20: Linearity graph of Vilanterol

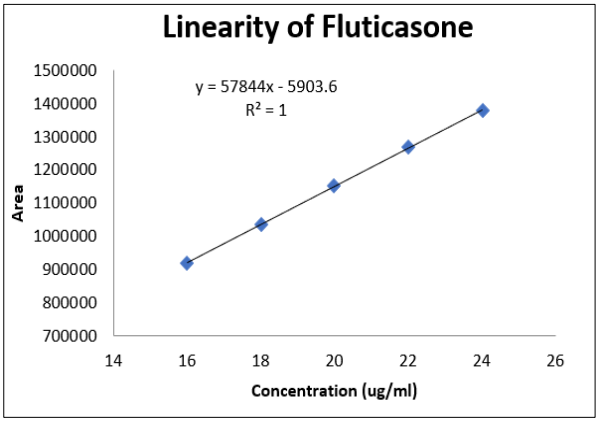


Figure 21: Linearity graph of Fluticasone Furoate

Table 11: Linearity data for Fluticasone Furoate

Fluticasone Furoate		
% Level	**Conc (ug/ml)**	**Area**
80	16	919574
90	18	1035411
100	20	1150809
110	22	1266755
120	24	1382343

From the above data it was found that the correlation coefficient of Vilanterol and Fluticasone Furoate were found to be 1 for both the Drugs. The Linearity graphs of Vilanterol and Fluticasone Furoate are given below:

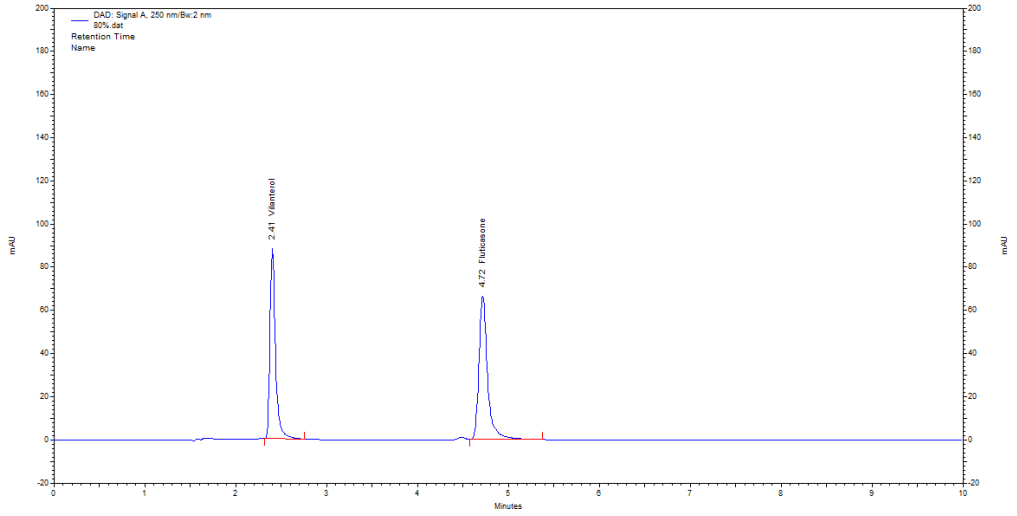


Figure 13: Linearity graph at 80%

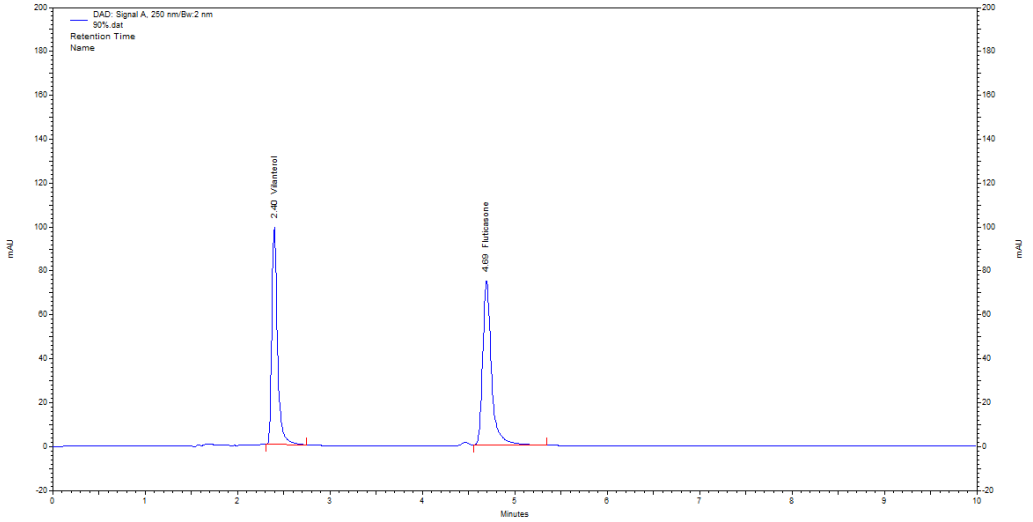


Figure 14: Linearity graph at 90%

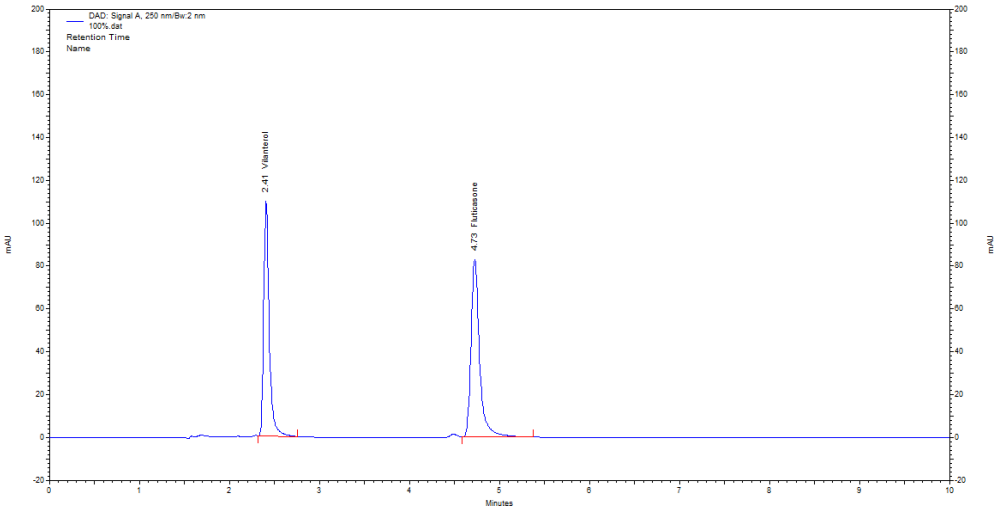


Figure 14: Linearity graph at 100%

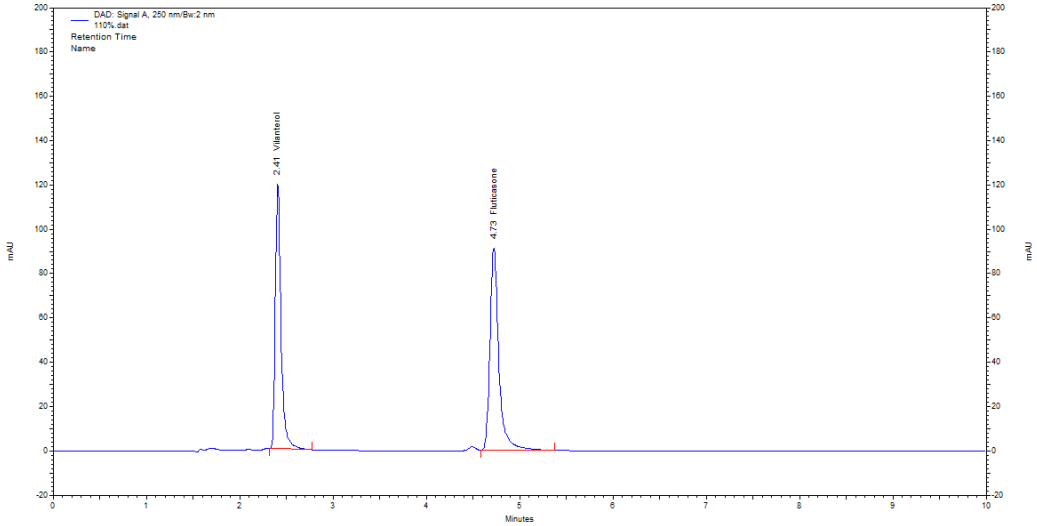


Figure 15: Linearity graph at 110%

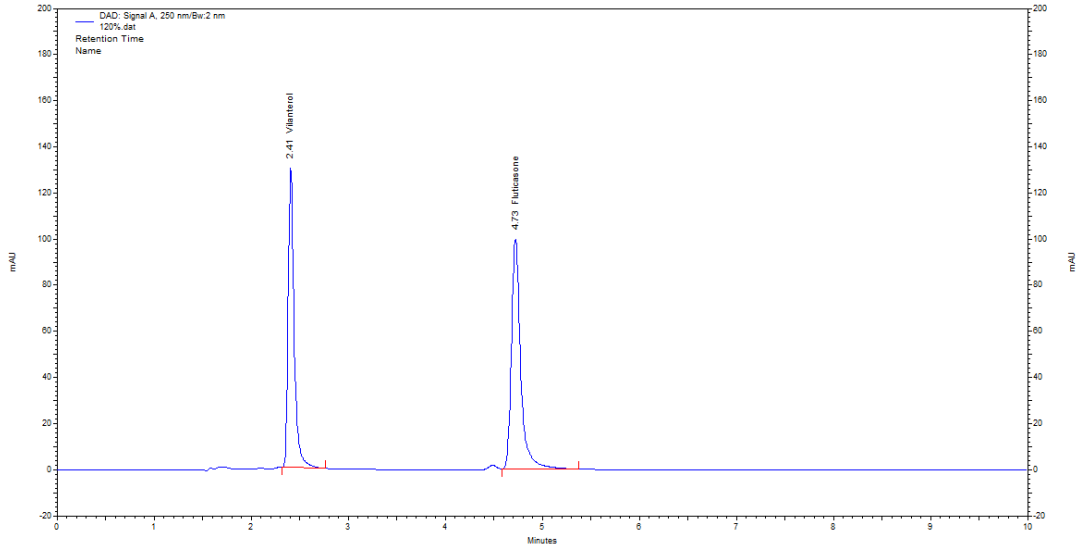


Figure 16: Linearity graph at 120%

IV.LOQ (Limit of Quantification) /LOD (Limit of Detection)

For Vilanterol, the limit of detection (LOD) and limit of quantification (LOQ) were established to be 0. 03 µg/ml and 0. 09 µg/ml, respectively. The regression analysis revealed high linearity with the coefficient of determination (R^2) of 0. 999990289, which means that the data fits very well to the regression model. The results of ANOVA also supported the model (F = 308938. 035, p < 0. 00001). Likewise, for Fluticasone Furoate, the LOD and LOQ were determined to be 0. 02 µg/ml and 0. 07 µg/ml, respectively. The regression analysis showed high correlation with the value of R^2 equal to 0. 999999611, which indicates a very high degree of correspondence between concentration and response. The regression model was also highly significant as revealed by the ANOVA results (F = 7717422. 34, p < 0. 00001).

V.Accuracy

Accuracy assessments for Vilanterol and Fluticasone Furoate were conducted at three concentration levels, evaluating % recovery and %RSD based on three replicate injections per level. For Vilanterol, the %RSD values were remarkably low across all levels: 0.17% at 80%, 0.12% at 100%, and 0.05% at 120%. These results indicate excellent precision, as they all fall comfortably below the acceptance criterion of 2% for %RSD in replicate injections.

Regarding % recovery for Vilanterol, values were consistently high: 100.23% at 80%, 99.94% at 100%, and 100.01% at 120%. These figures demonstrate the method's ability to accurately recover Vilanterol from spiked samples across a range of concentrations. Moving to Fluticasone Furoate, detailed % recovery and %RSD data are provided in Table 13, showing similar precision and accuracy evaluations across its respective concentration levels.

Table 12: Accuracy for Fluticasone Furoate

STD wt. (mg)	Purity (%)	Potency (ug/ml)
10	99.9	99.9

STD Area	1150234

Sample ID	Reps	Spiked Conc. (ug/ml)	Area	Amt Recovered (ug/ml)	% Recovery	Average	STDEV	%RSD
80%	Rep 1	15.984	919574	15.97	99.93	99.93	0.008134	0.01
	Rep 2	15.984	919512	15.97	99.93			
	Rep 3	15.984	919425	15.97	99.92			
100%	Rep 1	19.98	1150809	19.99	100.05	100.14	0.286482	0.29
	Rep 2	19.98	1149125	19.96	99.90			
	Rep 3	19.98	1155485	20.07	100.46			
120%	Rep 1	23.976	1382343	24.01	100.15	99.77	0.263984	0.26
	Rep 2	23.976	1374512	23.88	99.58			
	Rep 3	23.976	1379665	23.97	99.96			

The %RSD of three replicates of Fluticasone Furoate for accuracy level 80%, 100% and 120% was found to be 0.01%, 0.29% and 0.26% respectively.
The % recoveries for accuracy level 80%, 100% and 120% was found to be 99.93%, 100.14% and 99.77% respectively. The chromatograms for accuracy are given below:

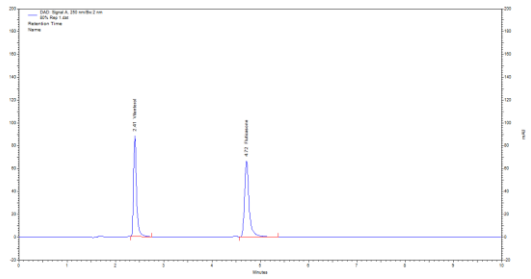


Figure 17: Accuracy Level- 80% Rep 1

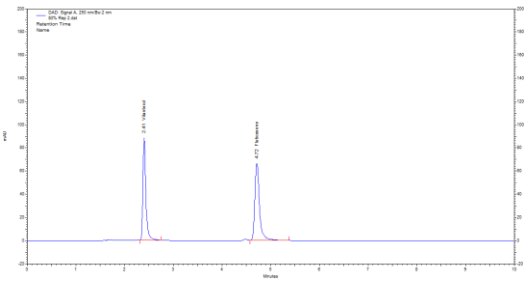


Figure 18: Accuracy Level- 80% Rep 2

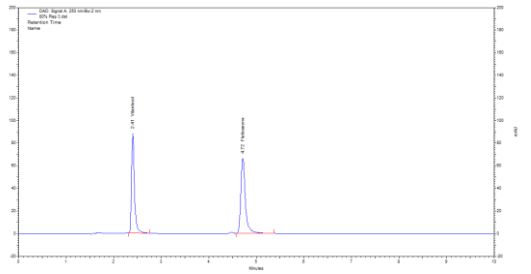


Figure 19: Accuracy Level- 80% Rep 3

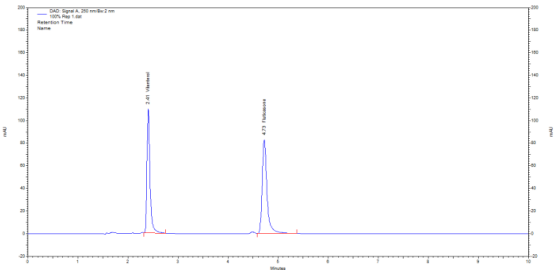


Figure 20: Accuracy Level- 100% Rep 1

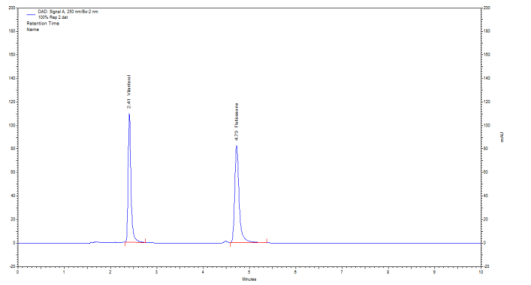


Figure 21: Accuracy Level- 100% Rep 2

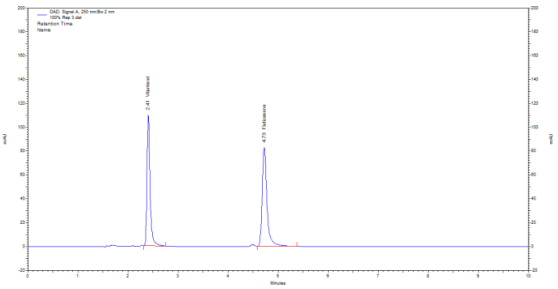


Figure 22: Accuracy Level- 100% Rep 3

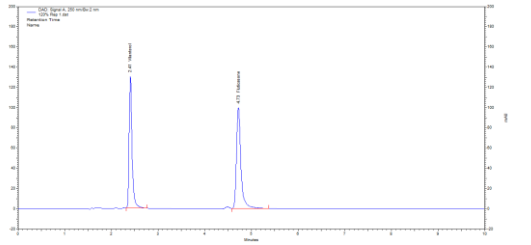


Figure 23: Accuracy Level- 120% Rep 1

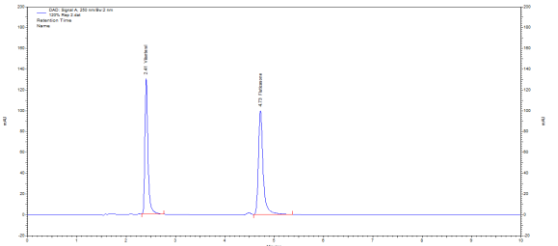


Figure 24: Accuracy Level- 120% Rep 2

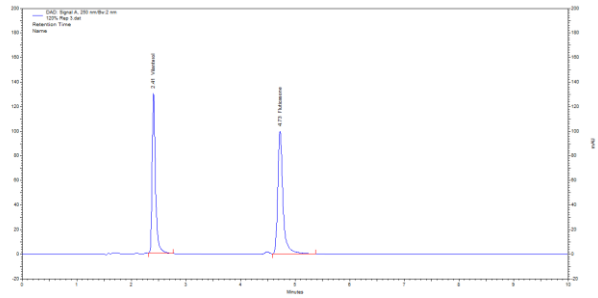


Figure 25: Accuracy Level- 120% Rep 3

VI.Robustness Study

Robustness study was done change in column temperature and change in Concentration of Mobile phase A (0.1% Perchloric acid).

Table 13: Robustness study - Change in Column temperature

Column Oven Temp Change							
Condition	**Sample**	**Vilanterol**			**Fluticasone Furoate**		
		RT	**Area**	**%Assay**	**RT**	**Area**	**%Assay**
28°C	**WS**	2.41	953365	-	4.73	1125687	-
	DP	2.41	954123	100.08	4.73	1122558	99.72
30°C	**WS**	2.41	974715	-	4.73	1150809	-
	DP	2.41	972533	99.78	4.73	1147854	99.74
32°C	**WS**	2.41	982211	-	4.73	1175210	-
	DP	2.41	980745	99.85	4.73	1171120	99.65

The Assay of Vilanterol at 28ºC, 30 ºC and 32 ºC was found to be 100.08%, 99.78% and 99.85% respectively. Whereas Fluticasone Furoate assay at 28ºC, 30 ºC and 32 ºC was found to be 99.72%, 99.74% and 99.65% respectively. The chromatograms are given below:

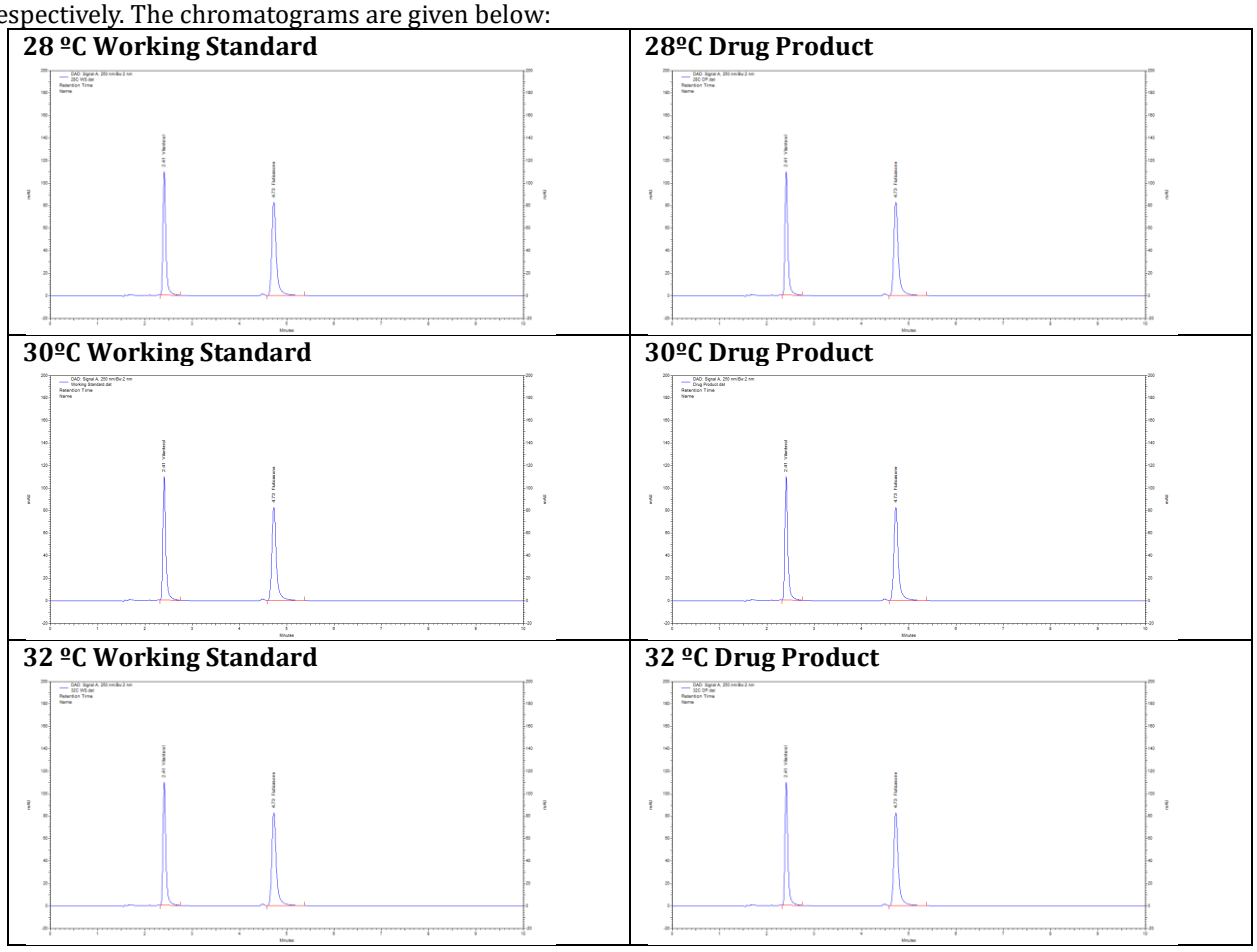


Figure 26: Robustness study- Column oven temperature

Table 14: Robustness study - Change in Concentration of Mobile phase A

Perchloric acid Concentration Change							
Condition	**Sample**	**Vilanterol**			**Fluticasone Furoate**		
		RT	**Area**	**Assay**	**RT**	**Area**	**Assay**
0.08%	**WS**	2.41	956966	-	4.73	1129654	-
	DP	2.41	951665	99.45	4.73	1125666	99.65
0.10%	**WS**	2.41	974715	-	4.73	1150809	-
	DP	2.41	972533	99.78	4.73	1147854	99.74
0.12%	**WS**	2.41	976220	-	4.73	1162010	-
	DP	2.41	975108	99.89	4.73	1159456	99.78

The assay results for Vilanterol at 0.08%, 0.1%, and 0.12% were 99.45%, 99.78%, and 99.89% respectively. For Fluticasone Furoate, the assay results at the same concentration levels were 99.65%, 99.74%, and 99.78% respectively. Chromatograms for both substances are provided below:

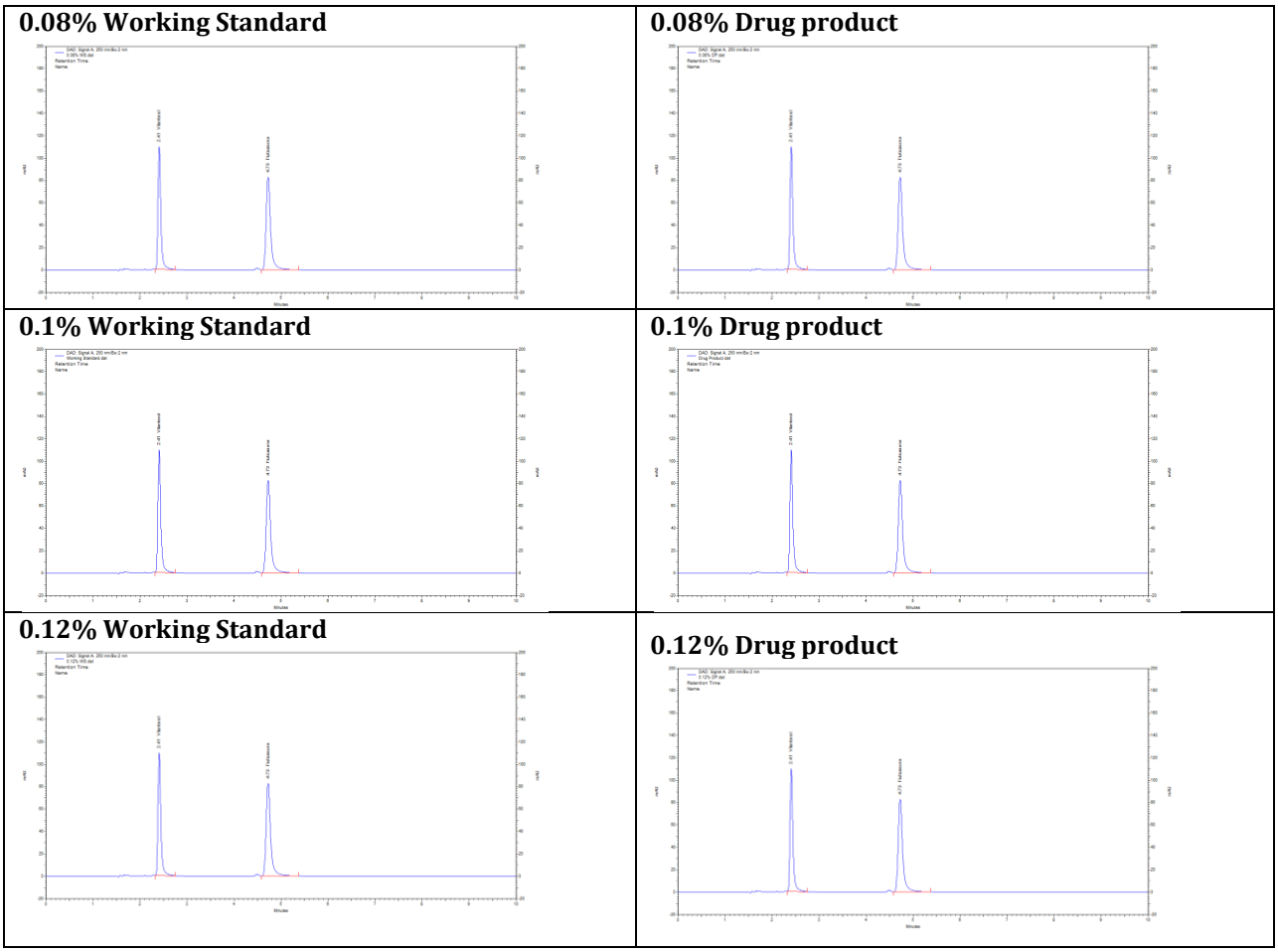


Figure 27: Robustness study- change in Mobile Phase A Concentration

Hence, the method was found to be robust with a small change in column temperature and change in Concentration of Mobile phase A. There was no significant change in Retention time, or Area of replicate injection.

VII.Intra and Inter day precision

Intra and inter day precision study was performed and reported the % RSD change in peak area of the APIs at different time points. The acceptance criteria is to have %RSD of peak area less than 2%

Table 15: Intra and Inter day Precision

Intra Day precision					
Day 1	**Sample ID**	**Vilanterol**		**Fluticasone Furoate**	
		Area	**Assay**	**Area**	**Assay**
Morning	**WS**	974715	-	1150809	-
	DP	972533	99.78	1147854	99.74
Evening	**WS**	964254	-	1136547	-
	DP	960105	99.57	1129896	99.41
		% RSD	0.15	**% RSD**	0.23
Inter Day precision					
Day	**Sample ID**	**Vilanterol**		**Fluticasone Furoate**	
		Area	**Assay**	**Area**	**Assay**
Day 2	**WS**	960202	-	1125658	-
	DP	955456	99.51	1117554	99.28
		% RSD	0.14	**% RSD**	0.24

The assay results for Vilanterol across morning, evening, and Day 2 were 99.78%, 99.57%, and 99.51% respectively. Similarly, for Fluticasone Furoate, the assay results were 99.74%, 99.41%, and 99.28% across the same time points. In terms of precision (%RSD), Vilanterol demonstrated 0.14% for both intra- and inter-day measurements, indicating consistent and reliable results. Fluticasone Furoate showed slightly higher %RSD values at 0.24%, maintaining acceptable precision across different days. Chromatograms illustrating intra- and inter-day precision are provided below:

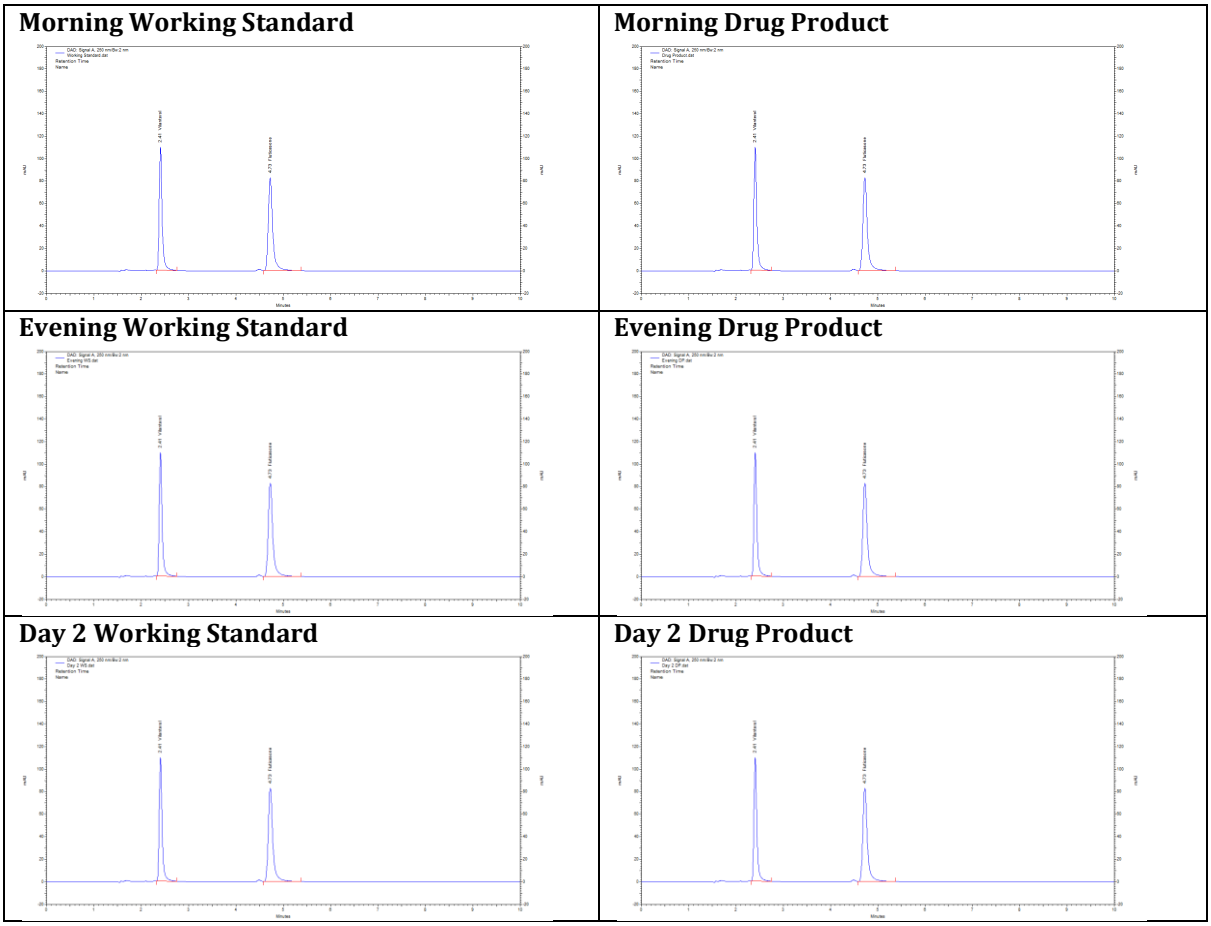


Figure 28: Intra and Inter day Precision

The working standard solution of Vilanterol and Fluticasone Furoate is stable for 2 days based on the % Assay of the APIs peak.

CONCLUSION

In the present study, a RP-HPLC method was developed and validated as per ICH Q2 (R1) guideline for the estimation of Vilanterol and Fluticasone Furoate in bulk and dosage form. The method used an Agilent Poroshell EC-120 C18 column with a mobile phase of 0. 1% perchloric acid and acetonitrile [35:65, operating at 0. The flow rate used was 8 ml/min and detection were done at 250 nm. Vilanterol and Fluticasone Furoate eluted at 2. 41 and 4. 73 minutes, respectively. Other parameters were the linearity ranges of 4-6 µg/ml for Vilanterol and 16-24 µg/ml for Fluticasone Furoate and the linearity was found to be excellent (r^2 = 1). The method had low LOD values (0. 03 µg/ml for Vilanterol, 0. 02 µg/ml for Fluticasone Furoate), and LOQ values of 0. 09 µg/ml and 0. 07 µg/ml, respectively. Precision studies showed %RSD values for Vilanterol instrument precision 0. 19%, intra-day precision 0. 14%, Fluticasone Furoate instrument precision 0. 29%, intra-day precision 0. 24%. Recovery was between 99 and 101% proving the reliability of the method for routine analysis because of the short time taken and lack of interference from excipients.

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