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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATIONOF BUDESONIDE AND FORMOTEROL IN BULK AND DOSAGE FORM USING RP-HPLC METHOD

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Abstract:

The present research work related to new method development of Formoterol Fumarate and Budesonide was found satisfactory, simple, precise, accurate with good resolution, shorter retention time and among the other degradation products both Formoterol Fumarate and Budesonide were well separated with all accurate results. Low limit of quantitation and limit of detection makes this method suitable for use in quality control. The less retention time obtained for the both drugs which reduces the run time enhances the usage of this method. This is the first reported method for stability indicting simultaneous quantitative analysis of Formoterol Fumarate and Budesonide, and is a significant advance in chromatographic analysis of such pharmaceutical mixtures.. Forced degradation study results have shown good separation from degradation peaks. Hence we can clearly say that the proposed method is Economic, stable and truly novel validated method than the other reported methods.In the present developed method the acceptable validation parameters makes this method of analysis more acceptable for the routine analysis in quality control department in industries approved testing laboratories,bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies.

Key Words: *Formoterol Fumarate , Budesonide, RP-HPLC.*

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INTRODUCTION:

Pharmaceutical Analysis is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation [1-4].

The technique employed in quantitative analysis is based upon the quantitative performance of suitable chemical reactions and either measuring the amount of reagent needed to complete the reaction, or ascertaining the amount of reaction product obtained.

Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no "second quality" in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production [5-7].

Physico-chemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the Physico-chemical methods, the most important are optical (Refractometry, Polarimetry, Emission, Fluorescence methods of analysis, Photometry including Photocolorimetry and Spectrophotometry covering UV-Visible and IR regions and Nephelometry or Turbidimetry) and chromatographic (Column, Paper, TLC, GLC, HPLC) methods. Methods such as Nuclear Magnetic Resonance and Para Magnetic Resonance are becoming more and more popular. The combination of Mass Spectroscopy with Gas Chromatography and Liquid Chromatography are the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis [8-10].

The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of pharmacopeia
3. The analysis should be economical.
4. The selected method should be precise and selective.

These requirements are met by the Physico-chemical methods of analysis, a merit of which is their universal nature that can be employed for analyzing organic compounds with a diverse structure. Of them, Visible Spectrophotometry is generally preferred especially by small scale industries as the cost of the equipment is less and the maintenance problems are minimal.

Introduction to Chromatography

Chromatography was originally developed by the Russian botanist Michael Tswett in 1903 for the separation of colored plant pigments by percolating a petroleum ether extract through a glass column packed with powdered calcium carbonate. It is now, in general, the most widely used separation technique in analytical chemistry having developed into a number of related but quite different forms that enable the components of complex mixtures of organic or inorganic components to be separated and quantified. A chromatographic separation involves the placing of a sample onto a liquid or solid stationary phase and passing a liquid or gaseous mobile phase through or over it, a process known as elution. Sample components, or solutes, whose distribution ratios between the two phases differ will migrate (be eluted) at different rates, and this differential rate of migration will lead to their separation over a period of time and distance.

Chromatographic techniques can be classified according to whether the separation takes place on a planar surface or in a column. They can be further subdivided into gas and liquid chromatography, and by the physical form, solid or liquid, of the stationary phase and the nature of the interactions of solutes with it, known as sorption mechanisms.

High Performance Liquid Chromatography:

In the modern pharmaceutical industry, HPLC is a major analytical tool applied at all stages of drug discovery, development and production. Fast and effective development of rugged analytical HPLC methods is more efficiently undertaken with a thorough understanding of HPLC principles, theory and instrumentation.

Liquid Chromatography (LC), which is one of the forms of Chromatography, is an analytical technique that is used to separate a mixture in solution into its individual components. The separation relies on the use of two different "phases" or "immiscible layers," one of which is held stationary while the other moves over it. Liquid Chromatography is the generic name used to describe any chromatographic procedure in which the mobile phase is a liquid. The separation occurs because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently relative to the other components in the mixture. HPLC is the term used to describe Liquid Chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector.

Validation:
Validation may be viewed as the establishment of an experimental data base that certifies an analytical method performs in the manner for which it was intended and is the responsibility of the method development laboratory. Method transfer, on the other hand, is the introduction of a validated method into a designated so that it can be used in the same capacity for which it was originally developed. .
Validation is defined as:
Food and Drug Administration (FDA):
Provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

Analytical method validation:
Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.
Before their introduction into routine use

- Whenever the conditions change for which the method has been validated, e.g., instruments with different characteristics.
- Whenever the method is changed, and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics.

The parameters as defined by the ICH and by other organizations are;

Precision:
"The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels; repeatability, intermediate precision and reproducibility."

Precision should be obtained preferably using authentic samples. As parameters, the standard deviation (SD), the relative standard deviation (coefficient of variation) and the confidence interval should be calculated for each level of precision.

Repeatability expresses the analytical variability under the same operating conditions over a short interval of time (within-assay, intra-assay). At least nine determinations covering the specified range or six determinations at 100 % test concentration should be performed. Intermediate precision includes the influence of additional random effects within laboratories, according to the intended use of the procedure, for example, different days, analysts or equipment, etc.
Reproducibility, i.e., the precision between laboratories (collaborative or interlaboratory Studies), is not required for submission, but can be taken into account for standardization of analytical procedures.

Specificity:
"Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedure(s)".

With respect to identification, discrimination between closely related compounds likely to be present should be demonstrated by positive and negative samples. In the case of chromatographic assay and impurity tests, available impurities / degradants can be spiked at appropriate levels to the corresponding matrix or else degraded samples can be used. For assay, it can be demonstrated that the result is unaffected by the spiked material. Impurities should be separated individually and/or from other matrix components. Specificity can also be demonstrated by verification of the result with an independent In the case of chromatographic separation, resolution factors should be obtained for critical separation. Tests for peak homogeneity, for example, by diode array detection (DAD) or mass spectrometry (MS) are recommended.

Accuracy:
"The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found".

Accuracy can be demonstrated by the following approaches:

- Inferred from precision, linearity and specificity
- Comparison of the results with those of a well characterized, independent procedure
- Application to a reference material (for drug substance)
- Recovery of drug substance spiked to placebo or drug product (for drug product)
- Recovery of the impurity spiked to drug substance or drug product (for impurities)

Linearity:
"The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample".

It may be demonstrated directly on the analyte, or on spiked samples using at least five concentrations over the whole working range. Besides a visual evaluation of the analyte signal as a function of the concentration, appropriate statistical calculations are recommended, such as a linear regression. The parameters slope and intercept, residual sum of squares and the coefficient of correlation should reported. A graphical presentation of the data and the residuals is recommended.

Range:
"The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity."
Limit of detection (LOD):

"The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest concentration of analyte in a sample which can be quantitatively determined with suitable precision and accuracy."

Various approaches can be applied:

- Visual definition
- Calculation from the signal-to-noise ratio (LOD and LOQ correspond to 3 or 2 and 10 times the noise level, respectively)
- Calculation from the standard deviation of the blank
  Calculation from the calibration line at low concentrations

LOD; LOQ ¼ F_SD b (2.6-1)

F: factor of 3.3 and 10 for LOD and LOQ, respectively

SD: standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression

b: slope of the regression line

The estimated limits should be verified by analyzing a suitable number of samples containing the analyte at the corresponding concentrations. The LOD or LOQ and the procedure used for determination, as well as relevant chromatograms, should be reported.

Limit of Quantification (LOQ):
The quantification limit is the lowest level of analyte that can be accurately and precisely measured. This limit is required only for impurity methods and is determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. If not determined experimentally, the quantification limit is often calculated as the analyte concentration that gives S / N = 10. An example of quantification limit criteria is that the limit will be defined as the lowest concentration level for which an RSD 20 % is obtained when an intra-assay precision study is performed.

Robustness:
According to ICH Q2A "the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage".
Furthermore, it is stated in ICH Q2B "The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used".

Ruggedness:
"The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different days, etc. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst". The degree of reproducibility is then evaluated by comparison of the results obtained

under varied conditions with those under standard conditions.

Materials:

FormoterolFumarate and Budesonide, KH_2PO_4, Water and Methanol for HPLC, Acetonitrile for HPLC, Ortho phosphoric Acid.

METHODOLOGY:

HPLC Method Development

Aliquots of the mixed solutions containing Formoterol Fumarate and Budesonide were prepared and a number of eluting experiments were conducted for the optimization of separation of drugs using mobile phase.

Optimized method

Mobile phase

Potassium dihydrogen phosphate buffer (pH-4.5), Acetonitrile were mixed in the ratio of 25: 75.It was filtered through 0.45 µm membrane filter and degassed20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column : Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase : Potassium dihydrogen phosphate buffer pH - 4.5) :Acetonitrile (30:70)
Elution mode : Isocratic.
Flow rate : 1 mL/min.
Detection wavelength : 280 nm.
Injection volume : 20.00 µL.
Run time : 10.00 min.
Retention Time : FF - 2.051 min.
BU - 4.234 min.

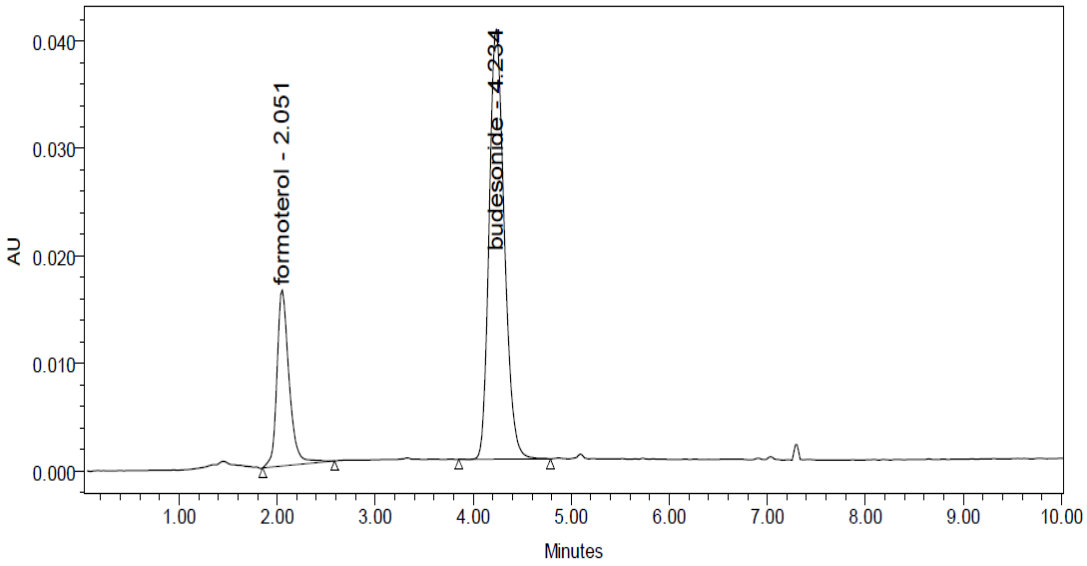

Fig.1: Chromatogram of optimized method

Table 1: Results of optimized method.

Drug	RT	Peak Area	TP	TF	Rs
FF	2.051	140672	2419	1.17	-
BU	4.234	427794	3591	1.21	8.6

Observation& Discussion*:*
The retention time was good for both FF and BU, the tailing factor was less than 2 and the number of theoretical plates were more than 2000 for both and the resolution was good, So this trail has been chosen as optimised method and further work was continued with this developed method that is validation.

Method Validation
Specificity:
Preparation of blank solution:
The Mobile phase, Potassium dihydrogen phosphate buffer pH - 4.5) :Acetonitrile (30:70) was taken as blank solution.
Preparation of standard solution*:*
Standard solution of Budesonide and Formoterol Fumarate and were prepared by dissolving 10 mg of each drug in 10 mL of mobile phase. Further dilution was made by adding 1 mL of the stock solution to 10 mL standard flask and making up the volume with the mobile phase.

Tablet sample preparation*:*
22 mg of Formoterol Fumarate and Budesonide capsule powder were accurately weighed and transferred into a 10 ml clean dry volumetric flask, 2 ml of diluent was added and sonicate to dissolve it completely and making volume up to the mark with the same solvent(Stock solution). Further pipette out 0.2 ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.
The above prepared solutions were injected and the chromatograms were recorded for the same.

Linearity:
Preparation of standard stock solution
Preparation of working standard solution
The working standard solution was prepared from the standard stock solution prepared. The prepared working standard solutions were injected and the chromatograms were recorded for the same.

Accuracy:
Accuracy of the method was determined by Recovery studies. To the formulation (preanalysed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100%, 150%.

Precision:
Precision was determined by analysing standard preparations of Formoterol Fumarateoxalate (50 µg/ mL) and Budesonide (2.5 µg/ mL)for six times.

Robustness:
Chromatographic conditions variation
To demonstrate the robustness of the method, prepared standard solution as per test method and injected in 5 replicate at different variable conditions like using different conditions like flow rate and temperature,wave length,mobile phase organic composition. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.System suitability parameters were compared with that of method precision

RESULTS:

Optimized method
Mobile phase
Potassium dihydrogen phosphate buffer (pH-4.5), Acetonitrile were mixed in the ratio of 25 : 75.It was filtered through 0.45 µm membrane filter and degassed20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column	:	Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase	:	Potassium dihydrogen phosphate buffer pH - 4.5) :Acetonitrile (30:70)
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength	:	280 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	FF - 2.051 min. BU - 4.234 min.

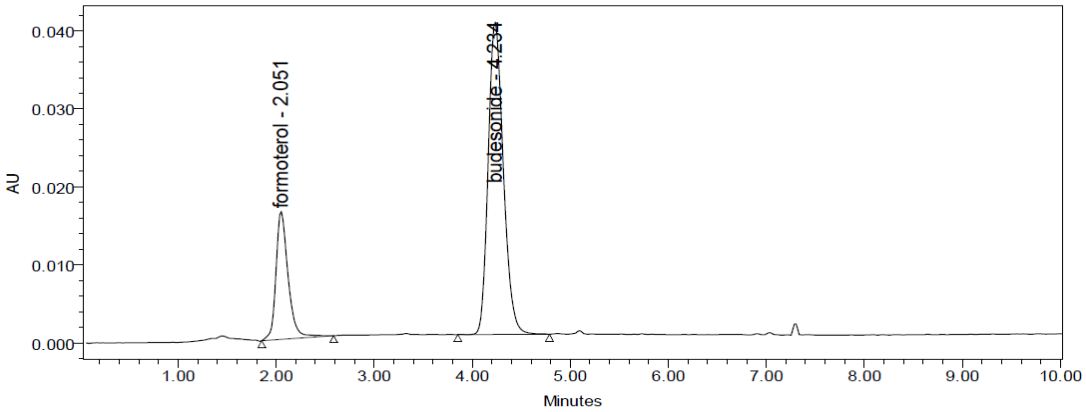

Fig 2: Chromatogram of optimized method

Validation Parameters

Specificity

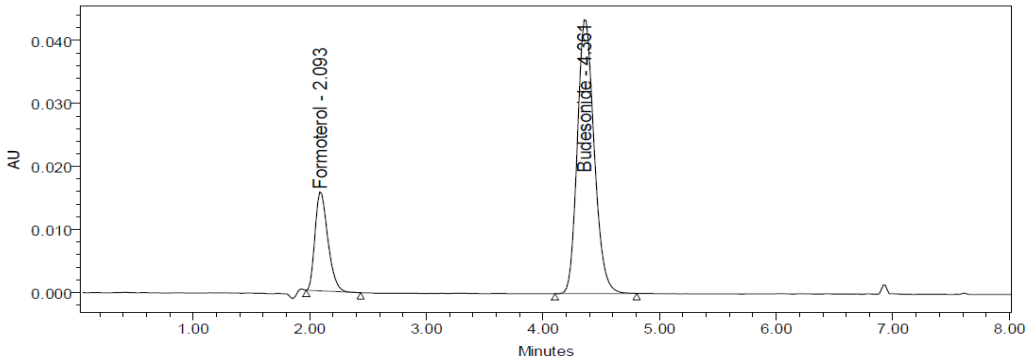

Fig 3: Chromatogram of Specificity

Table 2: For Specificity of Formoterol Fumarate and Budesonide

Drug	RT(min)	Peak Area	TF	Efficiency	Resolution
FF	2.093	120404	1.35	2729	-
BU	4.361	436416	1.19	4422	9.5

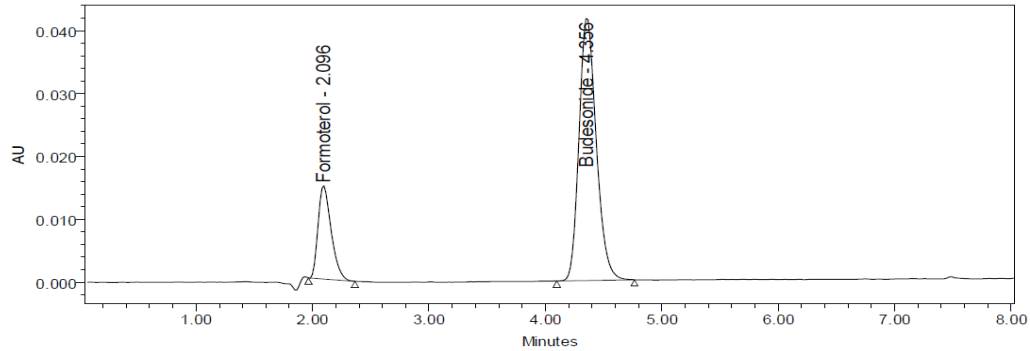

Fig. 4: Chromatogram for Specificity of FF and BUstandard

Table 3: Specificity of Formoterol Fumarate and Budesonide

Drug	Retention time (min)	Peak Area	TF	Efficiency	Resolution
FF	2.096	116063	1.40	2572	-
BU	4.356	428498	1.21	4211	9.2

Observation:
It was observed from the above data, diluent or excipient peaks are not interfering with the Formoterol Fumarate and Budesonide peaks.

Linearity

Table 4: Linearity Preparations

Preparations	Volume from standard stock transferred in ml	Volume made up in ml (with diluent)	Concentration of solution(µg /ml)	
			FF	BU
Preparation 1	0.3	10	30	1.5
Preparation 2	0.4	10	40	2
Preparation 3	0.5	10	50	2.5
Preparation 4	0.6	10	60	3
Preparation 5	0.7	10	70	3.5

Table 5: linearity data of Formoterol Fumarate and Budesonide

S.NO.	Conc(µg)	Area	Conc(µg)	Area
1.	15	76879	10	72549
2.	30	174699	20	578153
3.	45	110214	30	384068
4.	60	233585	40	726703
5.	75	344428	50	1075106

Accuracy

Table 6: Results for 50% Recovery

Injection	FF		BU	
	RT	Area	RT	Area
1	2.655	87403	5.781	278081
2	2.693	88028	5.932	285613
3	2.664	88863	5.835	282085
Avg	2.6706	88098	5.849	281926.33

Table 7: Results for 100% Recovery

Injection	FF		BU	
	RT	Area	RT	Area
1	2.738	183067	5.897	552361
2	2.387	151053	4.953	490353
3	2.207	136633	4.556	469094
Avg	2.444	156917.66	5.135	503939.33

Table 8: Results for 150% Recovery

Injection	FF		BU	
	RT	Area	RT	Area
1	2.148	196994	4.412	697285
2	2.148	196994	4.412	697285
3	2.435	235736	5.342	815472
Avg	2.243	209908	4.722	736680.666

Table 9: Results for Recovery of Formoterol fumarate

Concentration	Area	Amount added(µg/ mL)	Amount found(µg/ mL)	% Recovery	% mean Recovery
50	88098	5	49.26	70.8%	
100	156917.66	10	87.75	78..9%	
150	209908	15	117.38	99.9%	100%

Table 10: Results for Recovery of Budesonide

Concentration	Area	Amount added(µg/ mL)	Amount found(µg/ mL)	% Recovery	% mean Recovery
50	87088	5	46.24	69.8%	
100	158614.61	10	82.68	70.7%	
150	208809	15	110.28	89.9%	100%

Acceptance criteria

The % recoveryof Formoterol Fumarate and Budesonide should lie between 98.0% and 102.0%. The RSD of all the recovery values should not be more than 2.0%.

Observation

The percentage mean recovery of Formoterol Fumarate and Budesonide is and respectively and the results were found to be within the limits.

Precision

Table 11: Results for Precision.

Injection	Formoterol Fumarate		Budesonide	
	RT	Area	RT	Area
1	2.092	132443	4.327	436949
2	2.093	130445	4.330	435877
3	2.094	128713	4.331	431699
4	2.094	128211	4.332	432385
5	2.095	132105	4.333	433739
6	2.096	126517	4.333	435272
Average		1297389		434319.9
SD		2331.2		2058.8
%RSD		1.8		0.5

Robustness

Variation In Flow

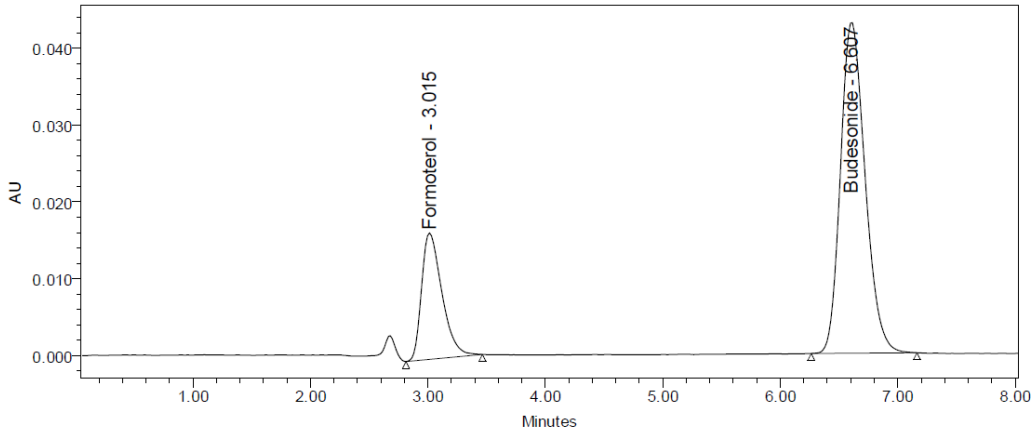

Fig 5: Chromatogram of FF and BUfor Robustness(Less flow 0.8 mL/min)

Table 12: Results of Formoterol Fumarate and Budesonidefor Robustness (0.8ml/min)

Drug	RT(min)	Peak Area	TF	Efficiency	Resolution
FF	3.015	197661	1.40	2436.50	-
BU	6.607	625443	1.21	4740.46	10.08

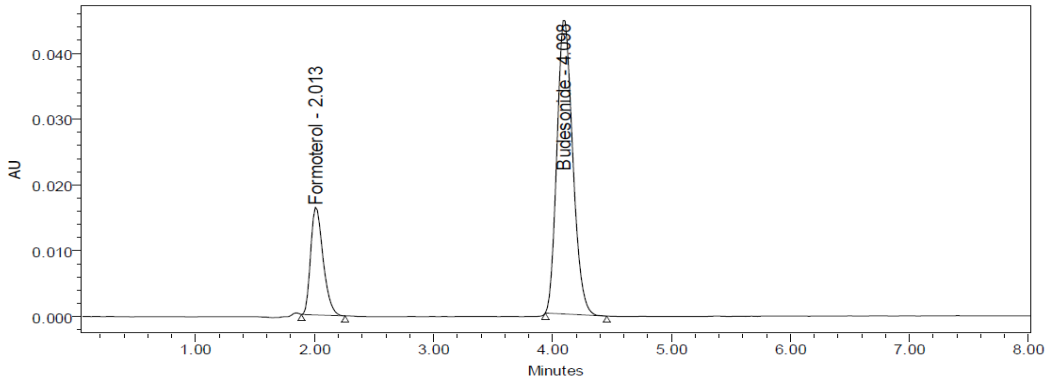

Fig.6: Chromatogram ofFormoterol Fumarate and Budesonide for Robustness (More flow 1.2 mL/min)

Table 13: Results of Formoterol Fumarate and Budesonide for Robustness (1.2 mL/min)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.013	118857	1.35	2750.35	-
BU	4.098	413383	1.21	44983	9031

Variation of mobile phase organic composition

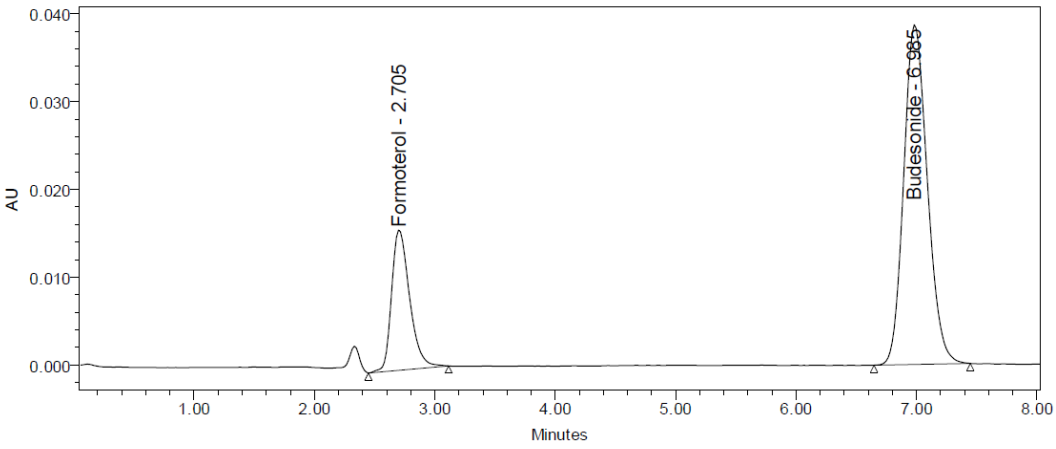

Fig.7: Chromatogram of Formoterol Fumarate and Budesonide for Robustness Less Organic

Table 14: Results of Formoterol Fumarate and Budesonide for Robustness (Less Organic)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.705	166234	1.28	1624.81	-
BU	6.985	527144	1.15	6001.97	13.38

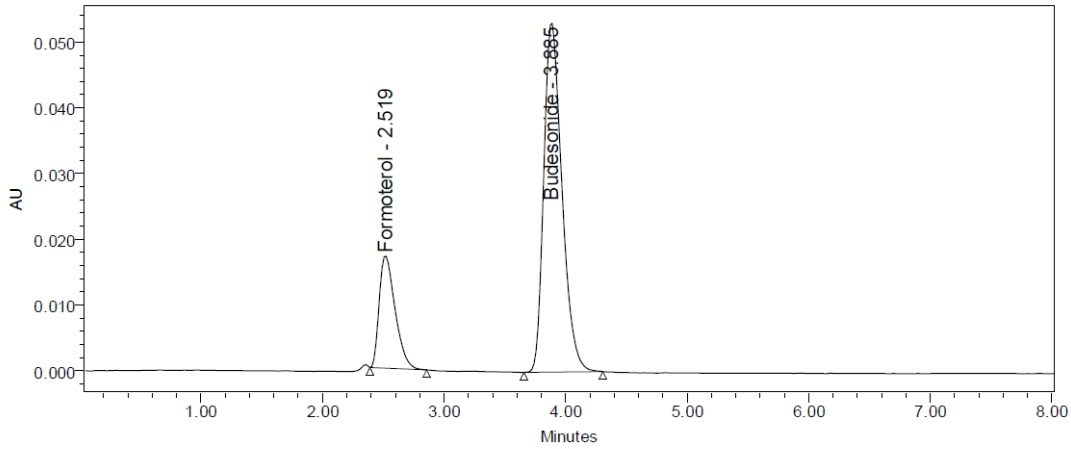

Fig.8: Chromatogram of Formoterol Fumarate and Budesonide forRobustness(More Organic)

Table 15: Results of Formoterol Fumarate and Budesonide for Robustness (More Organic)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.519	155106	1.49	2736067	-
BU	3.885	551579	1.31	3171014	5.18

Table16: Result of Robustness study

Parameter	Formoterol Fumarate (FF)		Budesonide (BU)	
	RT(min)	TF	RT(min)	TF
Flow Rate				
0.8 mL/min	3.015	1.40	6.607	1.21
1.0 mL/min	2.051	1.17	4.234	1.21
1.2 mL/min	2.013	1.35	4.098	1.21
Mobile Phase				
Less organic(55%) peak area	2.705	1.28	6.985	1.85
organic (50%) peak area	2.051	1.17	4.234	1.21
More organic (65%) peak area	2.519	1.49	3.885	1.31S

Observation

From the results of robustness by variations in flow rate and wavelength and composition of Mobile phase given in Table 8.37. It was observed that not much variation in tailing factor was observed with deliberate changes in flow rate and wavelength and composition of mobile phase. The tailing factor was found to be within the limits for Formoterol Fumarate and Budesonide .

SUMMARY AND CONCLUSION:

From the above observation data of the present research work related to new method development of FormoterolFumarate and Budesonide was found satisfactory, simple, precise, accurate with good resolution, shorter retention time and among the other degradation products both FormoterolFumarate and Budesonide were well separated with all accurate results. Low limit of quantitation and limit of detection makes this method suitable for use in quality control. The less retention time obtained for the both drugs which reduces the run time enhances the usage of this method.

This is the first reported method for stability indicting simultaneous quantitative analysis of FormoterolFumarate and Budesonide , and is a significant advance in chromatographic analysis of such pharmaceutical mixtures.. Forced degradation study results have shown good separation from degradation peaks. Hence we can clearly say that the proposed method is Economic, stable and truly novel validated method than the other reported methods

In the present developed method the acceptable validation parameters makes this method of analysis more acceptable for the routine analysis in quality control department in industries.Approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies.

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