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Research Article

Validation of *In vitro* Deposition of Emitted Dose Method for Simultaneous Estimation of Formoterol Fumarate and Glycopyrrolate in combined Dry Powder Inhaler Aerosol Pharmaceutical Dosage Form

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ABSTRACT

Formoterol fumarate and glycopyrrolate dry powder inhaler is a combined pharmaceutical aerosol dosage form. The label claim of this combined dosage form is 6 mcg of formoterol fumarate and 25 mcg of glycopyrrolate per respicap. It is prescribed for the treatment of asthma and chronic obstructive pulmonary disease. The deposition of an emitted dose is an *in vitro* investigational test that provides the idea of drug deposition inside the patient's lung. The present study aimed to validate the high performance liquid chromatography (HPLC) method to determine the deposition of the emitted dose of these analytes. This validation study was performed according to International Conference on Harmonization (ICH) guidelines and covers precision, linearity, accuracy, robustness, ruggedness, stability of analytical solution, and specificity. The reversed-phase chromatographic method uses a mobile phase prepared by mixing buffer containing sodium sulfate with ion pair reagent, acetonitrile, methanol, and purosphere star 125 mm column, 1 mL per minute flow rate, 215 nm wavelength, 25°C column oven, and 100 µL injection volume. The method found linear over the range of 10 to 150% of target concentration with a correlation coefficient of 0.9997 for formoterol and 0.9999 for glycopyrrolate. The recoveries obtained were 103.1 to 103.8% for formoterol and 97 to 100.1% for glycopyrrolate. The fine particle dose, mass balance, and % RSD were found well within the precision acceptance criteria. No interference found at the retention time of analyte peaks in blank, placebo, and degradation conditions describe method specificity. The method showed a successful application for the determination of formoterol fumarate and glycopyrrolate in dry powder inhaler pharmaceutical formulation.

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are types of obstructive lung disease. The main symptoms of COPD include shortness of breath and cough with sputum production. COPD is a progressive disease, which means it typically increases and becomes worse with time.

Formoterol fumarate is a bronchodilator that dilates the bronchi and bronchioles, decreases resistance in the respiratory airway, increases airflow to the lungs, and is used in the treatment of asthma.^[1-3]

Glycopyrrolate, also known as glycopyrronium bromide, is a quaternary ammonium compound that is an anticholinergic drug. Glycopyrrolate blocks muscarinic receptors, thus, inhibiting cholinergic transmission. It is used to inhibit salivation and excessive secretions of the respiratory tract preoperatively, the reversal of neuromuscular blockade, and peptic ulcer.^[4] Aerosol dosage form has many advantages over classical pharmaceutical dosage forms. Dry powder inhalers are administered by mouth, using a suitable device, and reach directly to the patients' lungs to give the desired local effect in terms of fast relief and easy breathing.^[5-7]

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Analytical method development and validation for dry powder inhaler formulation is challenging work as the dose range is very low in microgram levels. The present validation study showed a successful application for determining formoterol fumarate and glycopyrrolate in the concentration range of 0.06 to 0.88 µg/mL and 0.25 to 3.75 µg/mL, respectively.^[8-11] Very limited analytical methods have been reported in the literature to estimate formoterol fumarate and glycopyrrolate drugs in combined aerosol formulation, which includes different chromatographic techniques, mainly with gradient elution, long runtime, and more usage of solvents and hence, less cost-effective.^[12-14] The present investigation reports precise, linear, specific, rugged, and robust isocratic method with a short runtime, less usage of solvents, and hence, very much cost-effective for usage in the pharmaceutical industry and, guide future scientific researchers.

MATERIALS AND METHODS

Chemicals and Reagents

Formoterol fumarate dihydrate used for this study is manufactured by Vamsi Labs Limited. Glycopyrrolate used for this study is manufactured by Harman Phenochem Limited, and formoterol fumarate and glycopyrrolate respicaps are manufactured by Zydus Cadila Healthcare Limited. These standards, samples, and Respihaler Device for inhalation are provided by Zydus Cadila Healthcare Limited, Ahmedabad, India. The study was performed at Pharmaceutical Technology Centre, an analytical development laboratory of Zydus Cadila Healthcare Limited. Reagents sodium sulfate, 1-hexane sulfonic acid sodium salt monohydrate, sulfuric acid, methanol, and acetonitrile used is manufactured by Merck Limited.

Instrument and Chromatographic System

Chromatographic separation and quantification of the deposition of the emitted dose of formoterol fumarate and glycopyrrolate were performed using the Shimadzu and Agilent HPLC system. The systems have a quaternary solvent manager, a sample manager, and column oven, with ultraviolet (UV) and diode array detector. The output signal monitoring and processing were done using LC solution and Chromeleon software. The separation was

achieved on Purosphere Star RP 18e (125 × 4.6 mm × 5 µm) column. The isocratic liquid chromatographic method employs mobile phase prepared by adding 1.6 grams of sodium sulfate anhydrous, 0.5-gram of 1-hexane sulfonic acid sodium salt monohydrate, and 3 mL of dilute sulfuric acid (0.5-gram in 100 mL purified water) to 1,000 mL HPLC grade water, and dissolved with the help of shaking with a glass rod and ultrasonication.^[12] The solution was filtered through a 0.45 µm nylon filter and degas. This buffer solution, acetonitrile, and methanol are mixed in the ratio of 680:240:80 mL (68:24:8 %v/v/v). The mobile phase was pumped with a flow rate of 1 mL per minute. The column oven temperature was maintained at 25°C, and the injection volume was kept as 100 µL. The detection was monitored at a wavelength of 215 nm. The mixture of buffer solution and methanol in the ratio of 620:380 mL (62:38 %v/v) is used as a diluent. The structure of drug molecules is shown in Fig. 1.^[15-18]

Standard and Sample Preparation

Standard solution of formoterol fumarate and glycopyrrolate was prepared at a concentration of 0.6 and 2.5 µg/mL, respectively, by dissolving the appropriate amount of drug substance standard in the diluent. The sample was prepared using the following procedure; introduced 7 mL diluent into the upper and 30 mL diluent into the lower impingement chamber and connected all the assembly component parts, as shown in Fig. 2. Arranged assembly vertical and adequately supported, and the lower jet-spacer peg of the lower jet assembly touches the lower impingement chamber's bottom; connected a suitable vacuum pump and critical flow controller to the outlet of the apparatus. Adjusted the airflow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 liters per minute, attached mouthpiece to assembly. Took ten respicaps for testing. Placed one capsule inside Respihaler device, pierce it by pressing the blue press buttons on the device. Attached the device with assembly. Set time of 4 seconds to the critical flow controller and

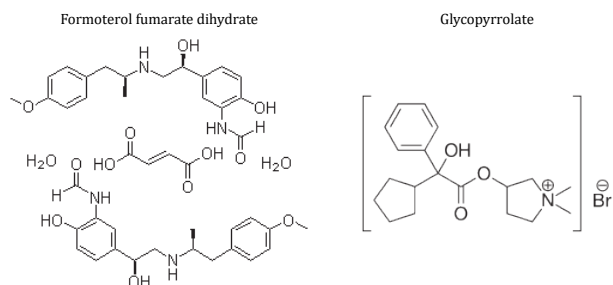


Fig. 1: Structure of drug molecules

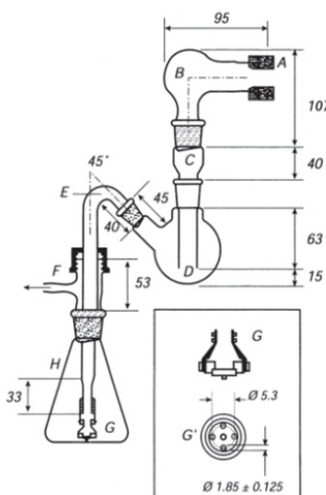


Fig. 2: Twin stage liquid impinger assembly



started the pump. After 4 seconds, the flow automatically stopped with the help of the critical flow controller, and the dose inside the capsule reached different parts of the assembly. Removed the empty capsule from the device. Now placed the second capsule inside Respihaler device, pierce and repeated procedure same as the first capsule. This procedure was performed for all 10 respicaps. Dismantled the apparatus, and samples should be prepared for upper impingement chamber T1 and lower impingement chamber T2.

Upper Impingement Chamber T1 Sample Preparation

Washed the mouthpiece and all parts of the upper impingement chamber with diluent and transferred into 100 mL volumetric flask with suitable funnel. Again rinsed all parts of upper impingement chamber and transferred solution into same 100 mL volumetric flask. Made volume up to a mark of 100 mL with diluent.

Lower Impingement Chamber T2 Sample Preparation

Washed the coupling tube and lower impingement chamber with diluent and transferred into 100 mL volumetric flask with the help of a suitable funnel. Again rinsed inner surfaces of lower impingement chamber and transferred solution into the same 100 mL volumetric flask. Made volume up to mark of 100 mL with diluent.

Method Validation

The developed analytical method was validated for its acceptable performance to ensure the suitability of the indent purpose. The validation parameters executed include accuracy, precision, specificity, linearity, range, ruggedness, and robustness experiments, per ICH guideline Q2 (R1).

System Suitability and System Precision

System suitability and system precision are determined by injecting multiple injections of the same standard

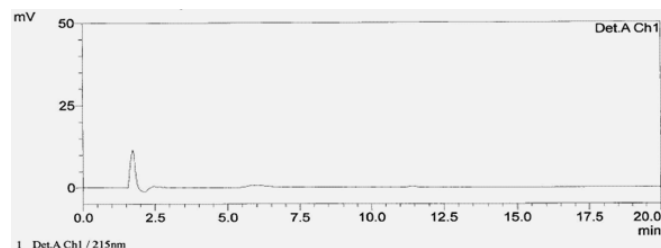


Fig. 3: Chromatogram of diluent

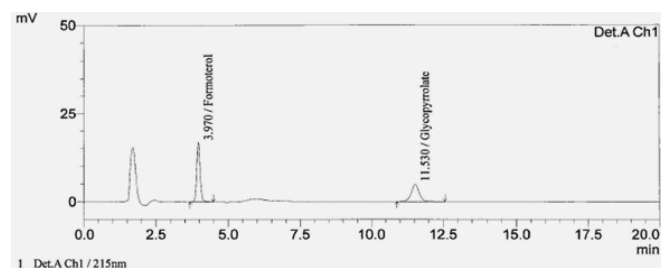


Fig. 4: Chromatogram of standard solution

preparation and calculating the % RSD for peak area of formoterol fumarate and glycopyrrolate. The theoretical plates and tailing factor were also recorded for formoterol fumarate and glycopyrrolate peaks.

The acceptance criteria were set to % RSD of multiple injections should not be more than 2%. The theoretical plates for formoterol and glycopyrrolate peaks should be not less than 1,500, and the tailing factor for each peak should be less than 2. Chromatograms of Diluent and standard preparation shown in Figs. 3 and 4, respectively.

Linearity and Range

The linearity of an analytical methodology is ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample matrix. Linearity was studied by analyzing the mixed calibration standard solutions at ten concentration levels. The linearity solutions were prepared from 10 to 150% level of target concentration for glycopyrrolate for formoterol fumarate. The Y-intercept, slope, and correlation coefficient were calculated for both analytes from the linear regression equation. The specification was set to the value of correlation coefficient R should not be less than 0.998 for all analytes. Y-intercept bias should not be more than 2% of the area of 100% linearity level. Linearity plot for absorbance vs. Concentration for formoterol fumarate and glycopyrrolate are shown in Figs. 5 and 6, respectively.

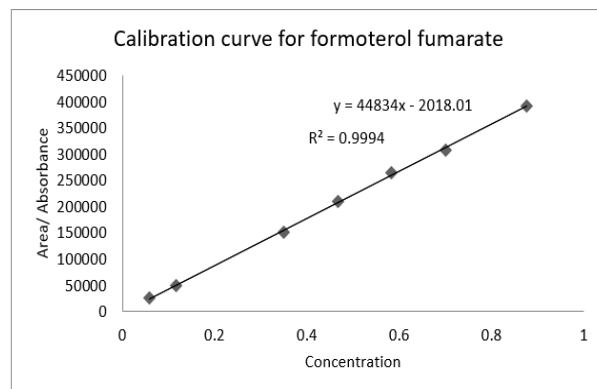


Fig. 5: Linearity plot for formoterol fumarate

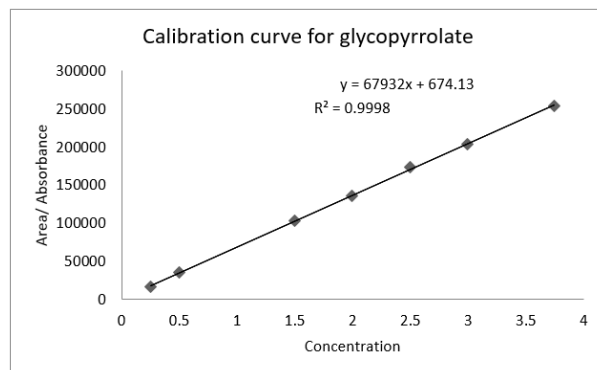


Fig. 6: Linearity plot for glycopyrrolate

Accuracy

The accuracy is sometimes also referred to as recovery. The accuracy can be defined as the closeness of the measured value (actual concentration) to the true value (theoretical concentration). Recovery is defined as how much drug was recovered from the added concentration using the proposed method.

Recovery was evaluated in triplicate by the addition of three sets of formoterol fumarate and glycopyrrolate standard to the formula placebo to get concentration at 10, 100, and 150% level of the target concentration. The specification set for accuracy is 95 to 105%, and % RSD should not be more than 5%.

Method Precision

Method precision study was performed by preparing ten same homogeneous sample preparations using a single batch. *In vitro*, fine particle dose and mass balance of formoterol fumarate and glycopyrrolate were calculated for all sample preparations. The mean of all results and percentage related standard deviation was also determined.

The specification was set for the percentage of the drug from lower impingement chamber T2 value for each individual set should not be less than 25%, and mass balance of all sample preparation should not be less than 85%.

Stability of Analytical Solutions

The stability of the analytical solution was determined by injecting standard and sample solutions at different time intervals up to 36 hours. The % RSD of the peak area of formoterol fumarate and glycopyrrolate was measured. The specification set to % RSD should not be more than 2% for both analytes.

Intermediate Precision (Ruggedness)

Intermediate precision expresses within-laboratories variations: different days, different columns, different equipment, etc. The study was performed by preparing ten same homogeneous sample preparations using a single batch. On a different day, by using a different lot of HPLC columns and by using different HPLC equipment. The specification was set the same as method precision study.

Robustness

The robustness of an analytical procedure is a measure of

Table 1: System suitability and system precision

S. No.	Parameters (n = 5)	Formoterol fumarate	Glycopyrrolate
1	Retention time (min)	4.02	11.62
2	Theoretical plates	4,634	7,295
3	Tailing factor	1	0.9
4	% RSD	0.1	0.2

its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It was studied by making the following variations in the method.

- The column oven temperature was changed to +5°C (20°C for -temperature condition and 30°C for +temperature condition).
- The flow rate was changed by +10% (0.9 mL per minute for -flow condition and 1.1 mL per minute for +flow condition).
- Mobile phase ratio was changed to +2 units absolute for buffer:acetonitrile:methanol (70:23:7 %v/v/v for -organic condition and 66:25:9 %v/v/v for +organic condition).

The system suitability criteria should be achieved for each changed condition during the robustness study.

Specificity

Specificity is the ability to assess the analytes unequivocally in the presence of components, which may expect to be present. Typically these might include placebo components, impurities, etc. The following solutions were prepared and injected into the HPLC system with a diode array detector to establish the specificity of the method.

- Mobile phase/ Diluent preparation
- Standard preparation
- Placebo preparation
- As such sample preparation (test preparation)
- Acid hydrolysis sample
- Alkali hydrolysis sample
- Peroxide oxidation sample
- Humidity degradation sample
- UV degradation sample

These samples were analyzed with a diode array detector, and the purity of analyte peaks was determined. The acceptance criteria were set to peak purity, should be greater than 990 for each peak. The degradation in the assay should be achieved between 10 to 30% in any one condition.

RESULTS

System Suitability and System Precision

System suitability and System precision data shown in Table 1. The Low percentage RSD value shows system is precise and suitable for estimation of Formoterol Fumarate and Glycopyrrolate.

Linearity and Range

Linearity data shown in Table 2, Y-intercept bias is found 0.76% for formoterol fumarate and 0.39% for glycopyrrolate with respect to the area of 100% linearity level indicates the method is linear.

Accuracy

As shown in Tables 3 and 4, Accuracy at each individual level was found between 95 to 105%, and % RSD was found



less than 5%. This indicates that the method is accurate over the range of 10 to 150% of the target concentration.

Method Precision (Repeatability)

As shown in Table 5, the percentage of the drug from the lower impingement chamber T2 value for each individual set is greater than 25%, and the mass balance of all sample preparation is higher than 85%. This indicates that the method is precise.

Stability of Analytical Solution

As shown in Tables 6 and 7, the % difference in the area at each level is less than 2%. The standard and sample

solutions are stable at room temperature for 36 hours.

Intermediate Precision (Ruggedness)

Intermediate precision data is shown in Tables 8 and 9. The same procedure followed as method precision but on different days, on different HPLC instruments and different column lot, using the same sample as specified under precision.

The absolute difference observed between method precision and intermediate precision is less than 5. % T2 of all individual sets, as well as, % mass balance also found well within the specified criteria with changing in the day, HPLC column, and HPLC instrument. This indicates that the method is rugged.

Table 2: Linearity data of formoterol fumarate and glycopyrrolate

Linearity level (%)	Final concentration in $\mu\text{g/mL}$ (formoterol fumarate)	Area	Final concentration in $\mu\text{g/mL}$ (glycopyrrolate)	Area
10	0.0584	25,423	0.2495	16,627
20	0.1167	50,038	0.499	34,734
60	0.3502	151,424	1.497	103,093
80	0.467	209,005	1.996	135,503
100	0.5837	264,059	2.495	172,799
100	0.7005	306,865	2.994	203,224
150	0.8756	392,268	3.7425	253,985

Table 3: Accuracy data of formoterol fumarate

For formoterol fumarate					
Level (%)	Amount of drug added (mg)	Amount of drug recovered (mg)	Recovery (%)	Mean (%)	% RSD
10	0.0117	0.0121	103.4	103.1	1.3
	0.0117	0.0122	104.3		
	0.0117	0.0119	101.7		
100	0.1169	0.1206	103.2	103.5	0.6
	0.1169	0.1206	103.2		
	0.1169	0.1218	104.2		
150	0.1816	0.1819	103.6	103.8	0.2
	0.1816	0.182	103.8		
	0.1816	0.1823	104		

Table 4: Accuracy data of glycopyrrolate

For glycopyrrolate					
Level (%)	Amount of drug added (mg)	Amount of drug recovered (mg)	Recovery (%)	Mean (%)	% RSD
10	0.0499	0.0499	100	100.1	0.9
	0.0499	0.0495	99.2		
	0.0499	0.0504	101		
100	0.499	0.4891	98	97.4	0.6
	0.499	0.4836	96.9		
	0.499	0.4855	97.3		
150	0.7485	0.7277	97.2	97	1.1
	0.7485	0.7335	98		
	0.7485	0.718	95.9		

Robustness

Robustness data is shown in Table 10. The RSD of five replicates of standard solution is less than 2% for all

conditions. The theoretical plates for each analyte peak are higher than 1,500, and the tailing factor for each analyte peak is less than 2. This indicates that the method is robust.

Table 5: Method precision data of formoterol fumarate and glycopyrrolate (TSLI)

Set no.	% T1		% T2		% mass balance	
	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate
1	61	60	50	45	111	105
2	59	61	40	48	99	109
3	57	62	44	49	101	111
4	58	62	48	49	106	111
5	62	64	48	47	110	111
6	64	67	50	44	114	111
Mean	60	63	47	47	107	110
% RSD	4.4	4	8.4	4.5	5.4	2.6

Table 6: Solution stability data for standard preparation

Time (hours)	Area		% difference from initial results	
	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate
Initial	283,925	195,372	-	-
6	283,456	196,111	0.1	0.3
12	283,568	195,856	0.1	0.2
18	283,654	195,788	0.1	0.2
24	283,384	195,410	0.1	0
30	283,396	195,632	0.1	0.1
36	283,211	195,469	0.2	0

Table 7: Solution stability data for sample preparation

Time (hours)	Area		% difference from initial results	
	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate
Initial	283,376	195,117	-	-
6	283,897	195,878	0	0.3
12	284,512	195,463	0.2	0.1
18	283,985	195,412	0	0.1
24	284,685	195,521	0.2	0.1
30	283,111	195,645	0.2	0.2
36	283,786	195,259	0	0.1

Table 8: Intermediate precision data of formoterol fumarate and glycopyrrolate (TSLI)

Set no.	% T1		% T2		% mass balance	
	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate	Formoterol fumarate	Glycopyrrolate
1	56	59	52	44	108	103
2	64	59	42	49	106	108
3	57	61	49	44	106	105
4	61	58	51	45	112	103
5	65	65	49	45	114	110
6	56	58	49	48	105	106
Mean	60	60	49	46	109	106
% RSD	6.8	4.5	7.2	4.7	3.4	2.8



Table 9: Comparison of method precision and intermediate precision

<i>Data comparison</i>	<i>Method precision</i>		<i>Intermediate precision</i>	
	<i>Day-1/ HPLC-1/ Column-1</i>		<i>Day-2/ HPLC-2/ Column-2</i>	
	<i>Formoterol fumarate</i>	<i>Glycopyrrolate</i>	<i>Formoterol fumarate</i>	<i>Glycopyrrolate</i>
% T2 mean	47	47	49	46
% mass balance	107	110	109	106
Absolute difference between assay results of method precision and intermediate precision	2		4	

Table 10: Robustness data

<i>For formoterol peak</i>							
<i>Condition</i>	<i>As such</i>	<i>+Flow</i>	<i>-Flow</i>	<i>+Temp</i>	<i>-Temp</i>	<i>+Org</i>	<i>-Org</i>
Area	109,877	100,071	121,236	109,583	109,624	109,534	108,404
Retention time	3.91	3.56	4.33	3.75	4.09	3.34	4.68
Theoretical plates	4,064	3,743	4,210	4,033	3,695	3,790	3,887
Tailing factor	1.1	1.1	1.1	1.1	1.1	1.1	1.1
<i>For glycopyrrolate peak</i>							
<i>Condition</i>	<i>As such</i>	<i>+Flow</i>	<i>-Flow</i>	<i>+Temp</i>	<i>-Temp</i>	<i>+Org</i>	<i>-Org</i>
Area	37,414	34,132	41,648	36,992	37,785	37,192	37,294
Retention time	10.62	9.66	11.75	10.14	11.07	8.2	13.93
Theoretical plates	6,208	5,683	6,090	5,911	5,491	5,426	5,555
Tailing factor	1	1	1	1	1.1	1.1	1.1

Table 11: Forced degradation data

<i>For formoterol fumarate</i>						
<i>Condition</i>	<i>As such</i>	<i>Acid deg.</i>	<i>Alkali deg.</i>	<i>Peroxide deg.</i>	<i>Humidity deg.</i>	<i>UV deg.</i>
% assay	99.3	75.3	97	94.2	95.3	95
% degradation	0	24	2.3	5.1	4	4.3
<i>For glycopyrrolate</i>						
% assay	100.3	89.1	82.5	98.2	98.2	98
% degradation	0	11.2	17.8	2.1	2.1	2.3

Specificity

The developed method's specificity was established by determining active components' peak purity in standard preparation and test preparation, using a photo diode array (PDA) detector. This is done using an assay sample and referred for deposition of emitted dose test.

There is no interference found at the retention time of analyte peaks in blank and placebo preparation. The purity of analyte peaks for all degradation conditions is greater than 990 for all degradation conditions. Data in Table 11 represents that formoterol is degraded in acid hydrolysis conditions. Glycopyrrolate degrades in alkali hydrolysis condition. The above observations indicate that the method is specific for its intended purpose.

DISCUSSION

During the literature review of different methods of aerosol dosage forms, previously reported work was found with gradient elution method having long runtime, higher solvent usage, and dual-wavelength for elution of these drugs.

In the present study, the isocratic reversed-phase HPLC method was developed and validated as per present ICH guidelines for the quantitative determination of *in vitro* deposition of emitted dose for formoterol fumarate and glycopyrrolate dry powder inhaler aerosol pharmaceutical dosage form. We used sodium sulfate combined with ion exchanger salt 1-hexane sulfonic acid sodium and sulfuric acid for this study. By optimizing the solvent ratio with these reagents, we achieved retention of formoterol peak at about 4 minutes and glycopyrrolate peak at about 11 minutes with theoretical plates more than 4,000 and tailing factor about one, for both analyte peaks. Hence, we could complete the method in 20 minutes runtime with isocratic elution.

There was a challenge of getting an optimum response. The label claim of this dry powder inhalation dosage form used for this study is very low (25 micrograms for glycopyrrolate and 6 micrograms for formoterol fumarate per respicap). We achieved an optimum area response by optimizing a single wavelength of 215 nm. Linearity and accuracy study also performed for very

vast concentration range, 10 to 150% of 0.6 µg/mL for formoterol fumarate, and 2.5 µg/mL for glycopyrrolate and achieved sound results, which makes the method more attractive, cost-effective, and can be utilized for different concentrations.

CONCLUSION

Based on all the above validation data obtained, it can be concluded that the developed method is simple, robust, specific, precise, rugged, accurate, and linear within the performed range and is very much suitable for its intended purpose. This validated method can be useful for combined estimation of formoterol fumarate and glycopyrrolate analytes in multiple industrial, as well as, academic applications. The detailed illustration of all present study parameters will also be useful to research scholars for easy understanding of validation practices.

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