International Journal of Pharmacy and Analytical Research (IJPAR)

ISSN: 2320-2831

IJPAR |Vol.12 | Issue 3 | July - Sept -2023
www.ijpar.com

Research article *Pharmaceutical Analysis*

Validated rp-hplc method development for the simultaneous estimation of glycopyrrolate and formoterol in its combined dosage forms

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Published on: September 5, 2023

ABSTRACT

A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of Glycopyrrolate and Formoterol in bulk and pharmaceutical formulations. Separation of Glycopyrrolate and Formoterol was successfully achieved on a Phenomenex Luna C18 (4.6×250mm, 5µm) particle size or equivalent in an isocratic mode utilizing Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v) at a flow rate of 1.0mL/min and eluates was monitored at 245nm, with a retention time of 2.102 and 3.537 minutes for Glycopyrrolate and Formoterol respectively. The method was validated and the response was found to be linear in the drug concentration range of 10µg/mL to 50µg/mL for Glycopyrrolate and 20µg/mL to 100µg/mL for Formoterol. The values of the slope and the correlation coefficient were found to be 77824 and 0.999 for Glycopyrrolate and 10515 and 0.999 for Formoterol respectively. The LOD and LOQ for Glycopyrrolate were found to be 0.6µg/mL and 1.8µg/mL respectively. The LOD and LOQ for Formoterol were found to be 0.8 µg/mL and 2.4µg/mL respectively. This method was found to be good percentage recovery for Glycopyrrolate and Formoterol were found to be 100.351 and 100.93 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analytes in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, Specificity and Robustness.

Keywords: Glycopyrrolate and Formoterol, High performance liquid chromatography, Validation.

INTRODUCTION

Introduction to HPLC [1-28]

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development and production. It is ideal for the analysis of many drugs in both dosage forms and biological fluids due to its simplicity, high specificity and good sensitivity.

High Performance Liquid Chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing. The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-5 cm3/min. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.

Types of Chromatography [1]

Normal-phase chromatography

Mechanism: Retention by interaction with the polar surface of the stationary phase with polar parts of the sample molecules.
Stationary phase: SiO2, Al2O3, -NH2, -CN, -Diol, -NO2, etc.
Mobile phase: Heptane, hexane, cyclohexane, CHCl3, CH2Cl2, dioxane, methanol, etc.
Application: Separation of non-ionic, non-polar to medium polar substances. Disadvantage: Lack of reproducibility of retention times as water or protic organic solvents change the hydration state of the silica or alumina chromatographic media.

Reversed-phase chromatography

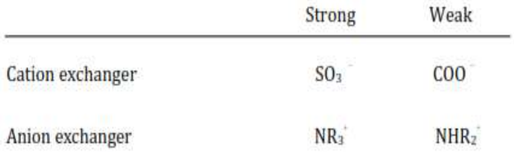
Mechanism: Retention by interaction of the stationary phase's non-polar hydrocarbon chain with non-polar parts of the sample molecules.
Stationary phase: n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, (CH2)n-CN, (CH2)n-diol, etc.
Mobile phase: Methanol, Acetonitrile, water, buffer (sometimes with additives of THF or Dioxane), etc.
Application: Separation of non-ionic and ion forming non-polar to medium polar substances (carboxylic acids, hydrocarbons). If ion forming substances (as carboxylic acids) are to be separated, a pH control by buffers is necessary.

Reversed-phase ion-pair chromatography

Mechanism: Ionic sample molecules are ionically bound to an ion-pair reagent. The ion- pair reagent contains an unpolar part suitable for interaction with the unpolar hydrocarbon chain of the stationary phase.
Stationary phase: Reversed phase materials (RP-18, RP-8, CN), etc.
Mobile phase: Methanol, Acetonitrile, buffer with added ion-pair reagent in the concentration range of 0.001 to 0.01 M, etc.
Application: Ionic substances often show very poor retention in reversed phase chromatography. To overcome this difficulty an ion-pair reagent is added to the eluent.

Ion-exchange chromatography

Mechanism: Retention of reversible ionic bonds on charged groups of the stationary phase
Stationary phase:



	Strong	Weak
Cation exchanger	SO_3^-	COO^-
Anion exchanger	NR_3^+	NHR_2^+

Mobile phase: Aqueous buffer systems.
Application: Separation of substances which can form ions such as inorganic ions, organic acids, organic bases, proteins, nucleic acids.

Advantages of HPLC [2]

1) It provides specific, sensitive and precise method for analysis of the different complicated sample.
2) There is ease of sample preparation and sample introduction.
3) There is speed of analysis.
4) The analysis by HPLC is specific, accurate and precise.
5) It offers advantage over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermo labile as well as nonvolatile substances.

Applications of HPLC [2]

a) Natural Products: HPLC is an ideal method for the estimation of various components in plant extracts which resemble in structure and thus demand a specific and very sensitive method e.g., analysis of digitalis, cinchona, liquorice, and ergot extracts.
b) Stability studies: HPLC is now used for ascertaining the stability of various pharmaceuticals. With HPLC the analysis of the various degradation products can be done and thus stability indicating HPLC systems have been developed.
c) Bioassays and its complementation: Complex molecules as antibiotics and peptide hormones are mainly analyzed by bioassay which suffers from high cost, necessity replicates, poor precision and length of time required. Also bioassay gives an overall estimate of potency and gives no guidance about the composition. Thus HPLC can be used to complement bioassays and give an activity profile. It has been used for analysis of chloramphenicol, penicillins and clotrimoxazole, sulfas and peptides hormones.
d) HPLC has also been used in the cosmetic industry for quality control of various cosmetics.

MATERIALS AND METHODS

Glycopyrrolate and Formoterol-Sura labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Acetonitrile for HPLC-Merck.

HPLC METHOD DEVELOPMENT TRAILS

Preparation of standard solution

Accurately weigh and transfer 10 mg of Glycopyrrolate and Formoterol working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.
Further pipette 0.1ml of the above Glycopyrrolate and 0.3ml of the Formoterol stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization

Initially the mobile phase tried was Methanol: Water and Water: Acetonitrile and Methanol: Phosphate Buffer: ACN with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Phosphate Buffer in proportion 45:55 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column, Symmetry and Zodiac column. Phenomenex Luna C18 (4.6×250mm, 5µm) particle size was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature : 35ºC
Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size
Buffer : Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 4.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra sonication.
pH : 4.6
Mobile phase : Acetonitrile: Phosphate Buffer (45:55 v/v)
Flow rate : 1ml/min
Wavelength : 245 nm
Injection volume : 10 µl
Run time : 7 min

Validation

Preparation of buffer and mobile phase

Preparation of Potassium dihydrogen Phosphate (KH2PO4) buffer (pH-4.6)

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 4.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra sonication.

Preparation of mobile phase

Accurately measured 450 ml (45%) of Methanol, 550 ml of Phosphate buffer (55%) were mixed and degassed in digital ultrasonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase: Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v)
Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size
Flow rate : 1 ml/min
Wavelength : 245 nm
Column temp : 35ºC
Injection Volume : 10 µl
Run time : 7 minutes

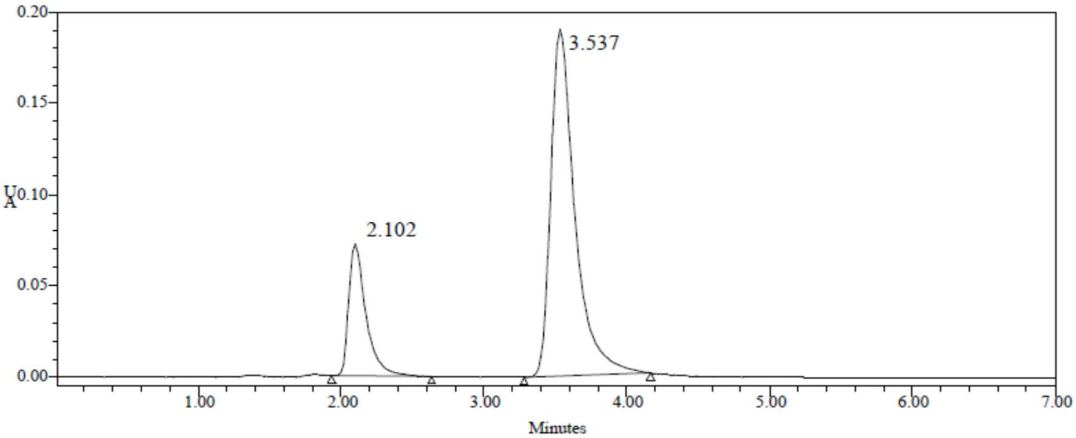


Fig 1: Optimized Chromatogram

Table 1: Peak results for Optimized Chromatogram

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Glycopyrrolate	2.102	765789	69584		0.97	5587.0
2	Formoterol	3.537	2532158	190049	2.97	1.26	5398.0

From the above chromatogram it was observed that the Glycopyrrolate and Formoterol peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

Mobile phase	: Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v)
Column	: Phenomenex Luna C18 (4.6×250mm, 5µm) particle size
Flow rate	: 1 ml/min
Wavelength	: 245 nm
Column temp	: 35ºC
Injection Volume	: 10 µl
Run time	: 7 minutes

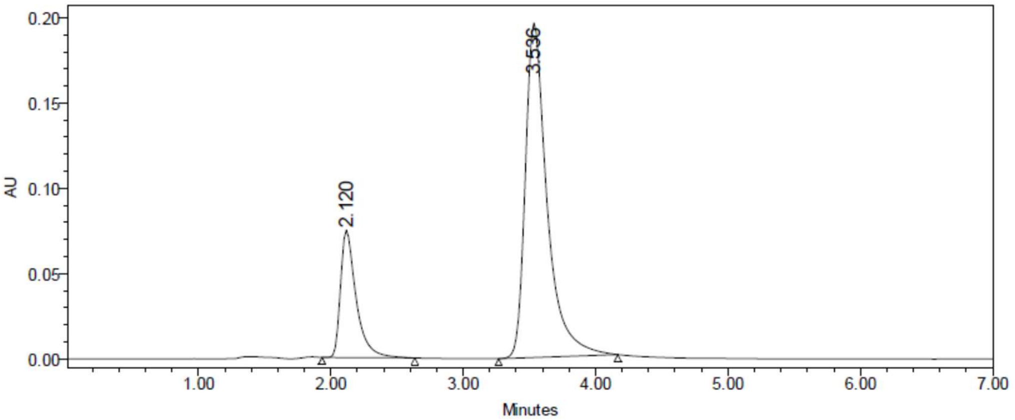


Fig 2: Optimized Chromatogram (Sample)

Table 2: Optimized Chromatogram (Sample)

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Glycopyrrolate	2.120	775684	13124		0.99	6365.0
2	Formoterol	3.536	2658478	937405	5.06	1.23	7458.0

- *Resolution between two drugs must be not less than 2.*
- *Theoretical plates must be not less than 2000.*
- *Tailing factor must be not less than 0.9 and not more than 2.*
- *It was found from above data that all the system suitability parameters for developed method were within the limit.*

Assay (Standard)

Table 3: Results of system suitability for Glycopyrrolate

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Glycopyrrolate	2.117	765843	69587	5589	1.9
2	Glycopyrrolate	2.118	766594	69854	5576	1.6
3	Glycopyrrolate	2.116	765487	70211	5658	1.6
4	Glycopyrrolate	2.109	765928	69213	5642	1.7
5	Glycopyrrolate	2.102	765426	69558	5685	1.6
Mean			765855.6			
Std. Dev			466.6522			
% RSD			0.060932			

- *%RSD of five different sample solutions should not more than 2*
- *The %RSD obtained is within the limit, hence the method is suitable.*

Table 4: Results of system suitability for Formoterol

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Formoterol	3.547	2534658	190058	5365	1.2	2.07
2	Formoterol	3.539	2536854	190052	5348	1.4	2.05
3	Formoterol	3.547	2535879	190078	5389	1.5	2.0
4	Formoterol	3.565	2533564	190035	5347	1.6	2.01

5	Formoterol	3.537	2534214	190085	5364	1.6	2.01
Mean			2535034				
Std. Dev			1183.309				
% RSD			0.046678				

- *%RSD for sample should be NMT 2.*
- *The %RSD for the standard solution is below 1, which is within the limits hence method is precise.*

Table 5: Peak results for assay standard

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Glycopyrrolate	2.102	759868	71255		1.7	5689	1
2	Formoterol	3.537	2458754	215654	2.04	1.6	5362	1
3	Glycopyrrolate	2.105	759458	72541		1.7	5748	2
4	Formoterol	3.552	2465885	226565	2.00	1.6	5452	2
5	Glycopyrrolate	2.112	759245	72584		1.7	5584	3
6	Formoterol	3.560	2489578	221542	2.04	1.6	5456	3

Table 6: Peak results for Assay sample

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Glycopyrrolate	2.120	756985	68958		0.98	7253	1
2	Formoterol	3.536	2569856	198564	2.06	1.23	8836	1
3	Glycopyrrolate	2.120	758745	69857		1.05	6530	2
4	Formoterol	3.537	2598654	195682	2.04	0.99	7270	2
5	Glycopyrrolate	2.102	756848	69588		1.7	7586	3
6	Formoterol	3.537	2587454	192541	2.04	1.6	8371	3

$$\%\text{ASSAY} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Glycopyrrolate and Formoterol in pharmaceutical dosage form was found to be 99.8%.

Data for linearity study

Glycopyrrolate

Concentration µg/ml	Average Peak Area
10	185689
20	349852
30	521541
40	685986
50	848265

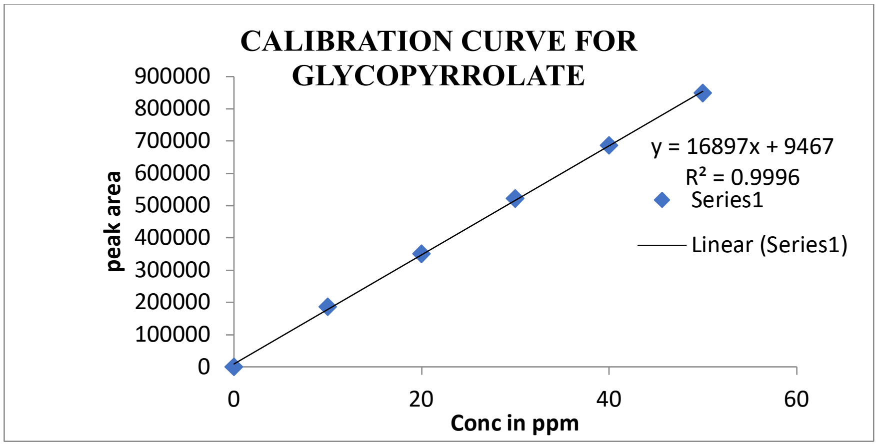


Fig 3: Calibration Graph for Glycopyrrolate

Formoterol

Concentration µg/ml	Average Peak Area
20	665985
40	1298698
60	1927852
80	2548545
100	3162468

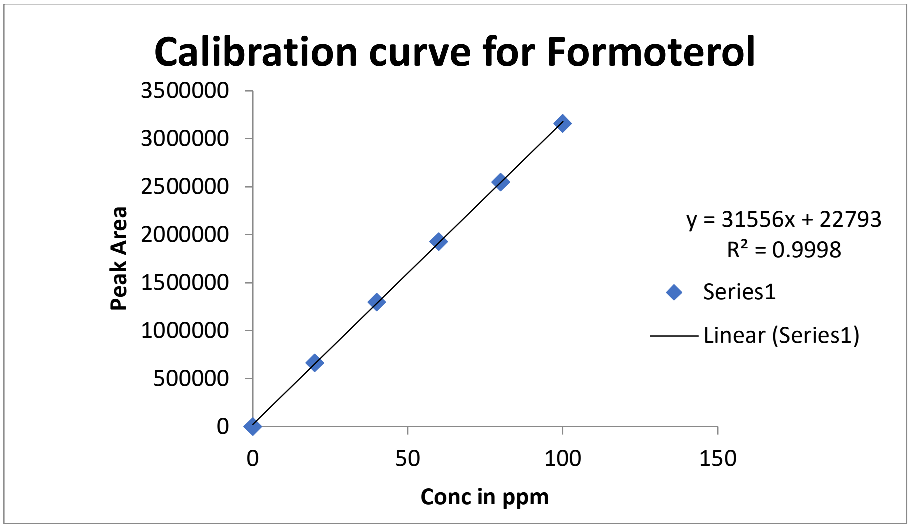


Fig 4: Calibration Graph for Formoterol

Repeatability

Table 7: Results of Repeatability for Glycopyrrolate

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Glycopyrrolate	2.108	766854	702564	5685	1.6
2	Glycopyrrolate	2.105	765884	698789	5584	1.4
3	Glycopyrrolate	2.113	765842	701235	5521	1.6
4	Glycopyrrolate	2.109	768985	700124	5525	1.9
5	Glycopyrrolate	2.109	765845	698986	5578	1.7
Mean			766682			

Std. Dev	1357.973
% RSD	0.177123

- *%RSD for sample should be NMT 2*
- *The %RSD for the standard solution is below 1, which is within the limits hence method is precise.*

Table 8: Results of method precision for Formoterol

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Formoterol	3.552	2569865	2231111	5365	1.6
2	Formoterol	3.550	2578474	2674210	5425	1.6
3	Formoterol	3.564	2568985	2231261	5368	1.5
4	Formoterol	3.564	2586845	2421301	5359	1.5
5	Formoterol	3.565	2545898	2324710	5498	1.6
Mean			2570013			
Std. Dev			15309.45			
% RSD			0.595695			

- *%RSD for sample should be NMT 2*
- *The %RSD for the standard solution is below 1, which is within the limits hence method is precise.*

Table 9: Results of Intermediate precision for Glycopyrrolate

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Glycopyrrolate	2.108	758955	68986	5785	1.6
2	Glycopyrrolate	2.105	759869	68957	5698	1.4
3	Glycopyrrolate	2.113	758985	68545	5689	1.6
4	Glycopyrrolate	2.109	756894	68952	5781	1.9
5	Glycopyrrolate	2.109	759854	68595	5785	1.7
6	Glycopyrrolate	2.102	756985	68952	5693	1.6
Mean			758590.3			
Std. Dev			1339.793			
% RSD			0.176616			

- *%RSD of Six different sample solutions should not more than 2.*

Table 10: Results of Intermediate precision for Formoterol

S.No.	**Name**	**Rt**	**Area**	**Height**	**USP plate count**	**USP Tailing**	**USP Resolution**
1	Formoterol	3.552	2659852	190025	5485	1.5	2.04
2	Formoterol	3.550	2648574	190048	5421	1.6	2.03
3	Formoterol	3.564	2659865	190054	5468	1.6	2.01
4	Formoterol	3.564	2658547	190078	5487	1.6	2.05
5	Formoterol	3.565	2648981	190016	5492	1.6	2.02
6	Formoterol	3.537	2654652	190057	5463	1.6	2.03
Mean			2655079				
Std. Dev			5242.086				
% RSD			0.197436				

- *%RSD of Six different sample solutions should not more than 2.*
- *The %RSD obtained is within the limit, hence the method is rugged.*

Table 11: Results of Intermediate precision Day 2 for Glycopyrrolate

Sno	**Name**	**Rt**	**Area**	**Height**	**USP plate count**	**USP Tailing**
1	Glycopyrrolate	2.102	766895	69858	5586	1.5
2	Glycopyrrolate	2.105	765988	69854	5636	1.6
3	Glycopyrrolate	2.112	766532	69824	5432	1.6
4	Glycopyrrolate	2.113	766214	69875	5468	1.6
5	Glycopyrrolate	2.109	765897	69854	5546	1.9
6	Glycopyrrolate	2.109	765245	69848	5507	1.7
Mean			766128.5			

Std. Dev	567.7234
% RSD	0.074103

%RSD of Six different sample solutions should not more than 2.

Table 12: Results of Intermediate precision for Formoterol

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Formoterol	3.537	2653254	190110	5428	1.6	7.98
2	Formoterol	3.552	2648985	190058	5452	1.6	6.4
3	Formoterol	3.560	2658213	190142	5498	1.6	8.9
4	Formoterol	3.564	2653652	190031	5442	1.5	8.3
5	Formoterol	3.564	2648978	190058	5489	1.5	7.5
6	Formoterol	3.565	2658985	190047	5463	1.6	5.3
Mean			2653678				
Std. Dev			4313.355				
% RSD			0.162543				

- *%RSD of Six different sample solutions should not more than 2*
- *The %RSD obtained is within the limit, hence the method is rugged.*

Accuracy

Table 13: The accuracy results for Glycopyrrolate

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	392891.7	5	5.027	100.540%	100.351%
100%	781996	10	10.026	100.260%	
150%	1171988	15	15.038	100.253%	

Table 14: The accuracy results for Formoterol

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	204962	15	15.156	101.040%	100.93%
100%	365018	30	30.378	101.260%	
150%	521064.3	45	45.218	100.484%	

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Robustness

Table 15: Results for Robustness

Glycopyrrolate

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical	Tailing factor
Actual Flow rate of 1.0 mL/min	765789	2.102	5587	1.7
Less Flow rate of 0.9 mL/min	758698	2.330	5458	1.7
More Flow rate of 1.1 mL/min	7689584	1.950	5696	1.7
Less organic phase	758412	2.290	5586	1.4
More organic phase	769852	1.998	5355	1.5

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Formoterol

Parameter used for sample analysis	Peak Area	Retention	Theoretical plates	Tailing
Actual Flow rate of 1.0 mL/min	2532158	3.537	5398	1.6
Less Flow rate of 0.9 mL/min	2458692	3.885	5329	1.7

More Flow rate of 1.1 mL/min	2658642	3.263	5256	1.7
Less organic phase	2452148	4.435	5214	1.2
More organic phase	2653894	3.009	5524	1.0

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION

A new method was established for simultaneous estimation of Glycopyrrolate and Formoterol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Glycopyrrolate and Formoterol by using Phenomenex Luna C18 (4.6×250mm, 5μm) particle size, flow rate was 1ml/min, mobile phase ratio was (45:55 v/v) Acetonitrile: Phosphate Buffer (pH-4.6 was adjusted with orthophosphoric acid), detection wave length was 245nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.102mins and 3.537mins. The % purity of Glycopyrrolate and Formoterol was found to be 99.8%. The system suitability parameters for Glycopyrrolate and Formoterol such as theoretical plates and tailing factor were found to be within limits. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study n Glycopyrrolate and Formoterol was found in concentration range of 10μg-50μg and 20μg-100μg and correlation coefficient (r^2) was found to be 0.999 and 0.999, % recovery was found to be 100.351% and 100.93%, %RSD for repeatability was 0.177 and 0.595. The precision study was precise, robust, and repeatable. LOD value was 0.6 and 0.8, and LOQ value was 1.8 and 2.4 respectively.

Hence the suggested RP-HPLC method can be used for routine analysis of Glycopyrrolate and Formoterol in API and Pharmaceutical dosage form.

ACKNOWLEDGEMENT

The authors are thankful to the Management and Principal, of the Department of Pharmacy, Smt Sarojini Ramulamma Colege Of Pharmacy, Mahabubnagar, for extending support to carry out the research work. Finally, the authors express their gratitude to the Sura Labs, Dilsukhnagar, Hyderabad, for providing research equipment and facilities.

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