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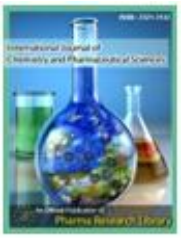

R E S E A E C H A R T I C L E

Analytical Method Validation Report for Essay of Glycopyrrocate and Formoteral Fumerate by RP-HPLC

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A B S T R A C T

A new method was established for simultaneous estimation of Glycopyrrolate and Formoterol fumerate by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Glycopyrrolate and Formoterol fumerate by using Xterra C18 (4.6 x 150mm, 5.0µm), flow rate was 1.0ml/min, detection wave length was 220nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, UV Detector, Empower-software version-2.The retention times were found to be 3.1 mins and 4.2 mins. The assay of Glycopyrrolate and Formoterol fumerate was performed with tablets and the % assay was found to be 99.80 and 99.72which shows that the method is useful for routine analysis. The linearity of Glycopyrrolate and Formoterol fumerate was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.6 for Glycopyrrolate and Formoterol fumerate which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.4 for Glycopyrrolate and Formoterol fumerate which shows that the method is repeatable when performed in different days also. The total recovery was found to be 100.01% and 100.34%for Glycopyrrolate and Formoterol fumerate. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The LOD and LOQ for Glycopyrrolate was found to be 3.02 and 3 and LOD and LOQ for Formoterol fumerate l was found to be 10 and 9.98The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits. Which shows that the method is having good system suitability and precision under given set of conditions.

Keywords: Glycopyrrolate, Formoterol fumerate, HPLC

A R T I C L E I N F O

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1. Introduction

Analytical methods

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness [1]. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available [2].

Description of the Various Analytical Methods

Titrimetric and gravimetric method of analysis is suitable when the sample is present in pure form or when no interference is observed in the mixture with other materials [3]. Ultraviolet and visible spectrometric method is suitable when no Interference is observed in the mixture [4,5]. HPLC and GC methods are more advantageous than the above due to their capability in separating organic mixtures and quantitative estimations. AAS is used mainly for quantitative estimation in ppm and ppb levels of elements Infra-red spectroscopy though mainly used for qualitative analysis can be used for quantitative estimation also. Out of all the above methods, thin layer chromatography plays a very important role in analysis due to its adaptability, flexibility, and cost and time. It can be used both for qualitative and quantitative determination. After separation spots can be scanned with the help of a scanner and quantitative measurement can be made [6].

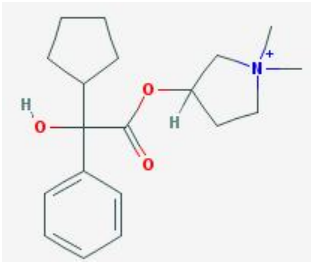

Figure 1: Glycopyrrolate

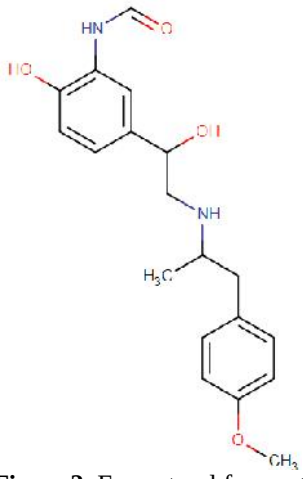

Figure 2: Formoterol fumarate

2. Materials and Methods

Apparatus: The instrument used for the study was WATERS, software: Empower, 2695 separation module, UV detector [10].

Mobile phase:

Accurately measured 500 ml (50%) of above buffer and 500 ml of Acetonitrile HPLC (50%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration [11]. Accurately measured 400 ml (40%) of above buffer and 600 ml of Acetonitrile HPLC (60%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtrationc.

Preparation of 0.1% OPA buffer:

Pipette out 1ml of Ortho Phosphoric Acid was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3 with NaOH

Diluent: The Mobile phase was used as the diluent.

Optimization Chromatographic trials for Simultaneous Estimation of Glycopyrrolate and Formoterol fumarate by RP- HPLC.

Optimization chromatographic conditions

Column : Xterra C18 (4.6 x 150mm, 5.0µm)
Mobile phase : 40% 0.1% OPA buffer pH3: 60% Methanol
Flow rate : 1.0 ml per min
Wavelength : 220 nm
Injection volume : 20 µl
Run time : 10min.

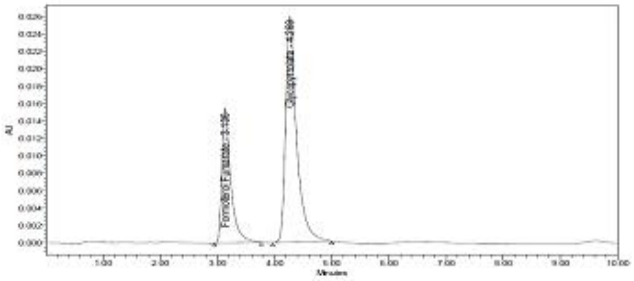

Figure 3: Optimization Chromatogram

Observation: The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

3. Results and Discussion

Method Validation Parameters

1. Linearity

The linearity study was performed for the concentration of 4.8 ppm to 24ppm for Glycopyrrolate and 9ppm to 45ppm for Formoterol fumarate and chromatograms are shown below.

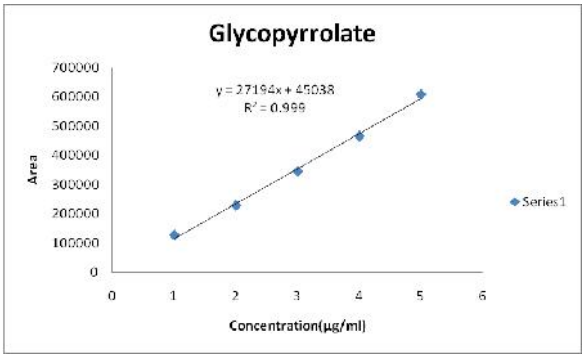

Figure 4: Calibration graph of Glycopyrrolate

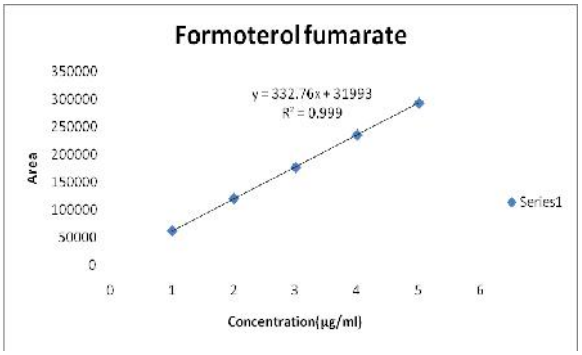

Figure 5: Calibration graph of Formoterol fumarate

2. Specificity:

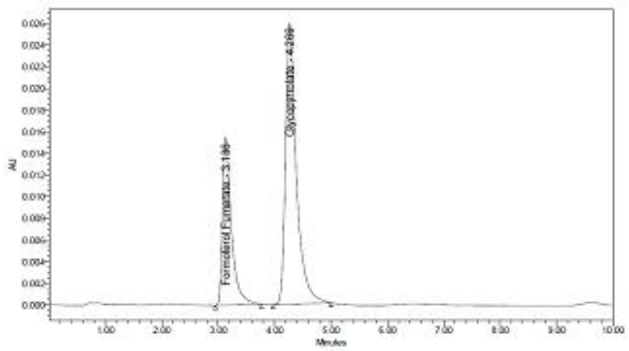

Figure 6: Chromatogram for System suitability

Preparation of stock solution:
Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent [12]. (Stock solution)

Preparation of Level – I:
0.1 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II:
0.2 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III:
0.3 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV:
0.4 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluents[13]

Preparation of Level – V:
0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure:
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient [14].

2. Precision:

Preparation of stock solution:
Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [15].

Procedure:
The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits [16].

3. Intermediate Precision/Ruggedness:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.

Preparation of stock solution:
Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [17].

Procedure:
The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits [18].

4. Accuracy:
For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of Standard stock solution [19]**:**
Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Preparation Sample solutions:
For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 4.5 mg of Glycopyrrolate and 2.4 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same

solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [20].

For preparation of 100% solution (With respect to target Assay concentration) [21]**:**

Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% solution (With respect to target Assay concentration) [22]**:**

Accurately weigh and transfer 13.6 mg of Glycopyrrolate and 7.2 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Procedure: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Glycopyrrolate & Formoterol fumarate and calculate the individual recovery and mean recovery values.[23]

5. Limit of Detection

Limit of Detection: (for Glycopyrrolate and Formoterol fumarate)

Preparation of 27µg/ml solution: Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [24].

Preparation of 0.21 µg/ml solution: Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents [25]. Further pipette 0.51ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

6. Limit of quantification:

For Glycopyrrolate and Formoterol fumarate)

Preparation of 27 µg/ml solution: Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [26].

Preparation of 0.68µg/ml solution:

Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [27]. Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.85ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

Preparation of 14.4µg/ml solution: Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [28].

Preparation of 0.66µg/ml solution: Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 4ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents [29].Further pipette 1.22ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

7. Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. A. The flow rate was varied at 0.9 ml/min to 1.1ml/min. Standard solution 14.4 ppm of Glycopyrrolate & 27 ppm of Formoterol fumarate was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly [30]. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The Organic composition in the Mobile phase was varied from 50% to 50%.

Standard solution 27ppm of Glycopyrrolate & 14.4ppm of Formoterol fumarate was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

8. Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active

substance. The aim of this work was to perform the stress degradation studies on the Glycopyrrolate and Formoterol fumarate using the proposed method [31].

Preparation of stock:

Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Hydrolytic degradation under acidic condition

Pipette 0.3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.3ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal induced degradation

Glycopyrrolate and Formoterol fumarate sample was taken in Petridis and kept in Hot air oven at 110^0 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analyzed [32].

Oxidative degradation

Pipette 0.3ml above stock solution into a 10ml volumetric flask and 1ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Table 1: Linearity Results: (for Glycopyrrolate)

S. No	Linearity Level	Concentration	Area
1	I	4.8	127774
2	II	9.6	228918
3	III	14.4	345340
4	IV	19.2	465502
5	V	24	607979
Correlation Coefficient			0.999

Table 2: Linearity Results: (for Formoterol fumarate)

S. No	Linearity Level	Concentration	Area
1	I	9	61241
2	II	18	119943
3	III	27	176636
4	IV	36	235363
5	V	45	293580
Correlation Coefficient			0.999

Table 3: Accuracy results for Glycopyrrolate

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	172505.0	4.5	4.47	99.38	100.01
100%	346412	9	8.98	99.78	
150%	525309.0	13.5	13.62	100.88	

Table 4: Accuracy results for Formoterol fumarate

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	85620	2.4	2.40	99.85	100.34
100%	171845	4.8	4.81	100.21	
150%	259676.0	7.2	7.27	100.95	

Table 5: Results of Precision for Formoterol fumerate and glycopyrrolate

Injection	Area for Glycopyrrolate	Area for Formoterol fumarate
Injection-1	341368	178876

Injection-2	340717	177224
Injection-3	342655	179055
Injection-4	343939	178739
Injection-5	343013	176699
Injection-6	342282	179220
Average	342329.0	178302.2
Standard Deviation	1156.8	1064.1
%RSD	0.3	0.6

Table 6: Results of Intermediate precision for Formoterol fumerate & Glycopyrrola

Injection	Area for Glycopyrrolate	Area for Formoterol fumarate
Injection-1	349453	172535
Injection-2	347162	171224
Injection-3	349458	172915
Injection-4	348377	173391
Injection-5	348482	173108
Injection-6	349771	172959
Average	348783.8	172688.7
Standard Deviation	976.1	769.7
%RSD	0.3	0.4

Table 7: Results for variation in flow for Glycopyrrolate:

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2452	1.12
2	1.0	2718.66	1.64
3	1.1	2255	1.22

Table 8: Results for variation in flow for Formoterol fumarate:

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2025.5	1.18
2	1.0	3961.26	1.15
3	1.1	2644.17	1.13

Table 9: Results for variation in mobile phase composition for Glycopyrrolate:

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2452	1.10
2	*Actual	2718.66	1.64
3	10% more	2055.73	1.13

Table 10: Results for variation in mobile phase composition for Formoterol fumarate:

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2025	1.18
2	*Actual	3961.26	1.15
3	10% more	3644	1.10

Table 11: Limit of Detection for Formoterol fumarate and Glycopyrrolate

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Formoterol fumerate	66	198	3.0

Glycopyrrolate	66	197	3.02

Table 12: Limit of Quantification for Formoterol fumarate and Glycopyrrolate

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Formoterol fumerate	66	659	9.98
Glycopyrrolate	66	660	10.00

Table 13: Degradation Studies of Glycopyrrolate

Sample Name	Glycopyrrolate	
	Area	% Degraded
Standard	346468.0	100
Acid	325453	93.93
Base	327849	94.63
Peroxide	325131	93.84
Thermal	328347	94.77
Photo	329359	95.06

Table 14: Degradation Studies of Formoterol fumerate

Sample Name	Formoterol fumarate	
	Area	% Degraded
Standard	171146.0	100
Acid	155289	90.73
Base	157420	91.98
Peroxide	163076	95.28
Thermal	163704	95.65
Photo	156820	91.63

4. Conclusion

A new method was established for simultaneous estimation of Glycopyrrolate and Formoterol fumerate by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Glycopyrrolate and Formoterol fumerate by using Xterra C18 (4.6 x 150mm, 5.0µm) , flow rate was 1.0ml/min, detection wave length was 220nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, UV Detector, Empower-software version-2.The retention times were found to be 3.1 mins and 4.2 mins. The assay of Glycopyrrolate and Formoterol fumerate was performed with tablets and the % assay was found to be 99.80 and 99.72which shows that the method is useful for routine analysis. The linearity of Glycopyrrolate and Formoterol fumerate was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.6 for Glycopyrrolate and Formoterol fumerate which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.4 for Glycopyrrolate and Formoterol fumerate which shows that the method is repeatable when performed in different days also. The total recovery was found to be 100.01% and 100.34%for Glycopyrrolate and Formoterol fumerate. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility.The LOD and LOQ for Glycopyrrolate was found to be 3.02 and 3 and LOD and LOQ for Formoterol fumerate l was found to be 10 and 9.98The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits. Which shows that the method is having good system suitability and precision under given set of conditions.

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