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Simultaneous Quantification of Formoterol Fumarate and Glycopyrrolate Using Reverse Phase High Performance Liquid Chromatography

ANANDAN KALAISELVAN[1,2,*], S.R. XAVIER RAJARATHINAM[1,2] and SARAVANAN CHANDRAN[1,2]

[1]Research and Development Centre, Bharathiar University, Coimbatore-641 046, India
[2]Department of Chemistry, Sacred Heart College (Autonomous), Tirupattur-635 601, India

*Corresponding author: E-mail: adr18phd@gmail.com

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A fully validated and rapid RP-HPLC method suitable for the quantification of formoterol fumarate and glycopyrrolate combination is reported. Chromatographic separation was achieved on a Sunsil C18 analytical column (250 mm × 4.6 mm, 5 µ particle size). The analytes were detected and quantified by photodiode array detector set at 289 nm. Calibration curves were constructed in the range of 4.8-14.4 µg/mL (R^2 = 0.9997) with a limit of quantification of 0.279 µg/mL for formoterol fumarate and 9-27 µg/mL (R^2 = 0.9998) with a limit of quantification 0.239 µg/mL for glycopyrrolate. The method was validated aaccording to ICH guidelines. The developed and validated RP-HPLC method was sensitive, selective, robust, accurate and precise for the simultaneous estimation of formoterol fumarate and glycopyrrolate in quality control laboratories.

Keywords: Formoterol fumarate, Glycopyrrolate, RP-HPLC.

INTRODUCTION

Formoterol fumarate is a β2 selective adrenoceptor agonist and produces bronchodilation in patients with obstructive airways disease [1,2]. Formoterol is principally used in the management of bronchial asthma and other types of allergic airway diseases. Chemically, it is known as (E)-but-2-enedioic acid; N-[2-hydroxy-5-[(1S)-1-hydroxy-2-[[(2S)-1-(4-methoxyphenyl) propan-2-yl]amino]ethyl]phenyl] formamide (Fig. 1).

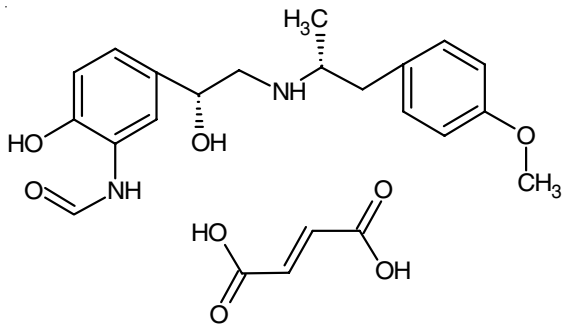

Fig. 1. Chemical structure of formoterol fumarate

Formoterol fumarate is an official drug in Indian Pharmacopoeia [3] and British Pharmacopoeia [4]. Formoterol fumarate was determined in bulk, pharmaceutical dosage forms, human plasma, serum and urine and rat plasma [5-18]. The techniques for the determination of formoterol fumarate include UV spectrophotometry [5], visible spectrophotometry [6,7], HPLC with UV detection [8-10], HPLC with electrochemical detection [11], HPTLC [12], HPLC-MS/MS [13-15], capillary electrophoresis [16,17], differential-pulse [18] and square-wave voltammetry [18].

Glycopyrrolate is a synthetic anticholinergic drug [19,20]. It also acts as an antispasmodic agent. Glycopyrrolate causes relaxation of smooth muscle and put off the occurrence of painful spasms. Glycopyrrolate also inhibits the release of gastric, pharyngeal, tracheal and bronchial secretions. Chemically, it is described as (1,1-dimethylpyrrolidin-1-ium-3-yl) 2-cyclopentyl-2-hydroxy-2-phenylacetate (Fig. 2).

Glycopyrrolate is an official drug in Indian Pharmacopoeia [21] and United States Pharmacopoeia [22]. HPLC with UV detection [23,24], HPTLC with densitometry [25], ESI-LC-MS/MS [26], UHPLC-MS/MS [27], UHPLC-HESI-MS-MS

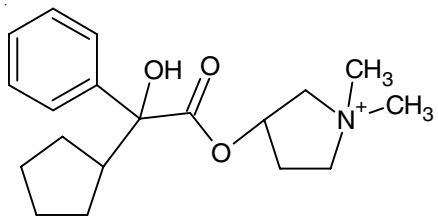

Fig. 2. Chemical structure of glycopyrrolate

[28], GC-MS [29] and capillary electrophoresis [30] techniques are found in the literature for the quantification of glycopyrrolate in bulk, pharmaceutical dosage forms, human plasma, horse urine and plasma and equine urine.

The formoterol fumarate and glycopyrrolate combination is used for the management of airflow obstacle in chronic obstructive pulmonary disease [31,32]. The studied drug combination is not official in any pharmacopeia. Till date, to the best of our literature review, only one RP-HPLC method has been reported for the simultaneous estimation of formoterol fumarate and glycopyrrolate [33]. Herein we report a sensitive and rapid quantification approach of formoterol fumarate and glycopyrrolate by means of RP-HPLC with photodiode array detection. The method was validated following ICH guidelines [34] and the method had been effectively used in routine analysis of studied drug combination.

The summary of the performance of reported and proposed RP-HPLC methods are shown in Table-1. The developed method has the advantages of sensitive, rapid (less run time), more precise and accurate than the reported method [33]. Furthermore reported method was not fully validated. The volume of sample used for analysis is less in proposed method (10 µL).

EXPERIMENTAL

Reference standard samples of glycopyrrolate and formeterol fumarate was kindly supplied by Lara drugs Pvt Ltd., Hyderabad. Acetonitrile of HPLC grade was obtained from Merck India Ltd., Mumbai. Potassium dihydrogen phosphate of analytical reagent grade was from Sd. Fine Chemicals Ltd., Mumbai. Purified water from a Milli-Q system was used in the analysis.

HPLC instrumentation and conditions: All analyses were done on a Waters HPLC system with a binary HPLC pumps model 2695, photodiode-array (PDA) detector model 2998 and a vacuum degasser. The HPLC system was controlled by Waters Empower2 software. The LC system was equipped with a Sunsil C18 column (250 mm, 4.6 mm, 5 µ particle size) and the separation and analysis were carried out at 25 °C. The mobile phase was 0.1 M dipotassium hydrogen phosphate and acetonitrile (60:40 v/v). A constant flow rate of 1 mL/min and injection volume of 10 µL was employed throughout the analyses. The photodiode array detector was set to 289 nm to detect and analyze the studied analytes.

Preparation of standard solutions: Mixed stock solutions of glycopyrrolate and formeterol fumarate was prepared by dissolving 9 mg (glycopyrrolate) and 4.8 mg (formeterol fumarate) in 100 mL of mobile phase in a 100 mL volumetric flask. Working standard solutions containing 9.0, 13.5, 18.0, 22.5 and 27.0 µg/mL glycopyrrolate and 4.8, 7.2, 9.6, 12.0 and 14.4 µg/mL formoterol fumarate were prepared by aptly diluting the stock solution with mobile phase. The glycopyrrolate and formoterol fumarate working standard solution with concentration 18.0 and 9.6 µg/mL, respectively was used for the study of validation parameters.

Preparation of placebo blank solution: 40 mg starch, 35 mg hydroxyl cellulose, 35 mg gum acacia, 20 mg lactose, 35 mg sodium citrate, 40 mg talc, 35 mg sodium alginate and 35 mg magnesium stearate were accurately weighed. All the common excipients were mixed into a homogeneous mixture. A 100 mg of the homogeneous mixture was accurately weighed and transferred to a 100 mL volumetric flask containing 30 mL of mobile phase. The contents of the flask were sonicated for 20 min and filtered using 0.45 µm membrane filter. The filtrate was diluted to 100 mL with mobile phase.

General assay procedure: 10 µL of working standard solutions in the concentration range 9-27 µg/mL (glycopyrrolate) and 4.8-14.4 µg/mL (formoterol fumarate) was injected into the HPLC system thrice. Using the described chromatographic conditions, the chromatograms and peak area response at each concentration were determined. A calibration curve was drawn with peak area response *vs.* drug concentration. The regression equation was derived using the obtained data. The concentration of unknown was determined either using the corresponding calibration curve or corresponding regression equation.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions: Two columns [YMC Pack pro C18 (250 × 4.6 mm; 5 µm particle size) column and Sunsil C18 (250 × 4.6 mm; 5 µm particle size) column] with different temperatures were compared by observing the resolution, tailing factor and plate count. Sunsil C18 (250 × 4.6 mm; 5 µm particle size) column with 25 °C yielded the best results. Hence the same column was selected. For mobile phase, 0.1 M potassium dihydrogen orthophosphate and acetonitrile were examined using diverse proportions, pH and flow rates. Good resolution between glycopyrrolate and formoterol fumarate drugs within short runtime was obtained by an isocratic elution using a mobile phase consisting of 0.1 M potassium dihydrogen orthophosphate and acetonitrile in the ratio 60:40 (v/v) at a flow rate of 1.0 mL/min with pH 4.5.

TABLE-1
PERFORMANCE OF REPORTED AND PROPOSED RP-HPLC METHODS

Drug	Run time (min)	Linearity (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	RSD (%)	Recovery (%)	Ref.
Formeterol fumarate	10	9-45	Not reported	Not reported	0.3-0.6	99.38-100.88	[33]
Glycopyrrolate		4.8-24	Not reported	Not reported	0.3	99.85-100.95	
Formeterol fumarate	6	4.8-14.4	0.084	0.279	0.24	99.62-99.73	Proposed method
Glycopyrrolate		9-27	0.072	0.239	0.12	99.97-100.22	

Detection and analysis of glycopyrrolate and formoterol fumarate was performed using photodiode array detector set at 289 nm. The chromatogram of glycopyrrolate and formoterol fumarate with retention times with optimized chromatographic conditions is shown in Fig. 3.

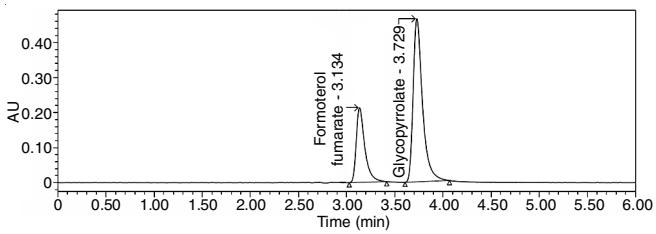

Fig. 3. Chromatogram of glycopyrrolate and formoterol fumarate obtained using optimized chromatographic conditions

Validation of assay method: The method was validated in accordance with the International Conference on Harmonization recommended guidelines [34] for system suitability, linearity, specificity, sensitivity, accuracy, precision and robustness.

System suitability: System suitability study is used to make sure that the reproducibility of the HPLC system is sufficient for the analysis to be done. Parameters including plate count, resolution, tailing factor and relative standard deviation for peak area response and retention time of drugs were calculated using glycopyrrolate and formoterol fumarate standard solution with concentration 18 and 9.6 µg/mL, respectively. The parameters required for system suitability test of the method are in acceptable limits as presented in Table-2.

Selectivity: Selectivity of the method was determined by comparing the chromatogram of standard drug (glycopyrrolate 18 µg/mL and formoterol fumarate 9.6 µg/mL) with chromatograms of placebo blank and mobile phase blank. The chromatograms of the same are presented (Fig. 4). No peaks were observed in the chromatograms of placebo blank and mobile phase blank. The results indicated that the common excipients and components of the mobile phase did not interfere with the detection and analysis of glycopyrrolate and formoterol fumarate. Hence the method is selective.

Linearity: Linearity was assessed by plotting the peak area response of drug against the concentration of drug using a simple least squares regression. The calibration curves were constructed by plotting the peak area *versus* the corresponding concentrations of drug in the range of 4.8-14.4 µg/mL for formoterol fumarate and 9-27 µg/mL for glycopyrrolate. The concentration of glycopyrrolate and formoterol fumarate was calculated from the following regression equation:

Formoterol fumarate: PA = 13499 C - 144.3 (R^2 = 0.9997)

Glycopyrrolate: PA = 17572 C + 2331 (R^2 = 0.9998)

where PA is peak area response, C is the concentration of drug in µg/mL and R^2 is the regression coefficient.

Sensitivity: Sensitivity of the method is assessed by determining limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ of the analytes were calculated using the following equations: LOD = 3 s/m and LOQ = 10 s/m. where 's' is the standard deviation of the peak area (five runs) of the standard drug, 'm' is the slope of the calibration curve. The calculated LOD was 0.084 and 0.072 and LOQ was 0.279 and 0.239 for formoterol fumarate and glycopyrrolate, respectively. The low values of LOD and LOQ indicated the sufficient sensitivity of the method for the assay of formoterol fumarate and glycopyrrolate.

TABLE-2
PARAMETERS OF SYSTEM SUITABILITY OF THE DEVELOPED METHOD FOR THE DETERMINATION OF GLYCOPYRROLATE AND FORMOTEROL FUMARATE

Injection No.	Formoterol fumarate					Glycopyrrolate				
	Retention time	Peak area	Plate count	Tailing factor	Resolution	Retention time	Peak area	Plate count	Tailing factor	Resolution
1	3.134	1297476	6547	1.57	-	3.729	3180379	7653	1.56	3.52
2	3.135	1291134	6604	1.54	-	3.73	3185516	7821	1.56	3.54
3	3.133	1286172	6598	1.55	-	3.728	3168626	7856	1.56	3.55
4	3.132	1324023	6512	1.59	-	3.725	3140517	7704	1.53	3.51
5	3.133	1290664	6633	1.52	-	3.725	3131232	7814	1.5	3.54
Mean	3.133	1297894	6579	1.554	-	3.727	3161254	7770	1.542	3.532
RSD (%)	0.036	1.167	0.737	1.739	-	0.062	0.765	1.114	1.740	0.465
Recommended limits	RSD ≤ 2	RSD ≤ 2	> 2000	≤ 2	-	RSD ≤ 2	RSD ≤ 2	> 2000	≤ 2	> 1.5

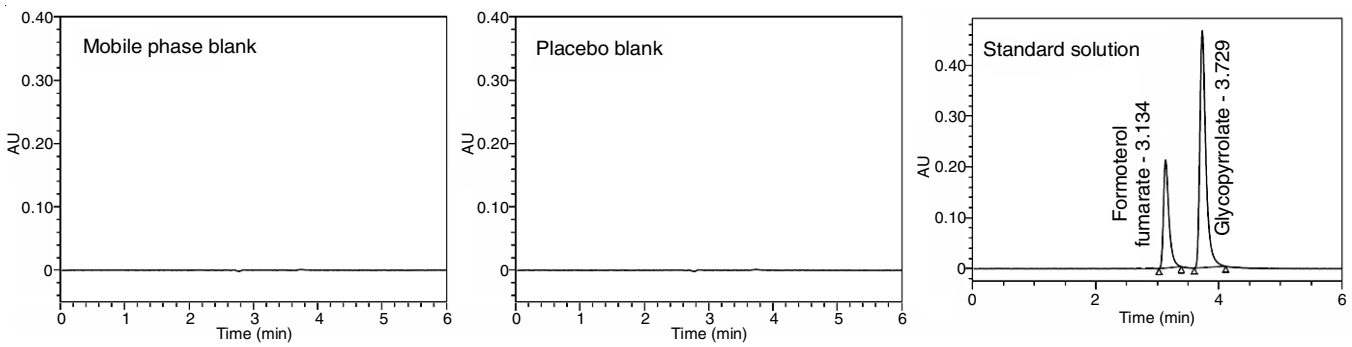

Fig. 4. Chromatograms of selectivity study

Precision and accuracy: Precision and accuracy were investigated with five replicates of standard drug solution (glycopyrrolate 18 µg/mL and formoterol fumarate 9.6 µg/mL). Precision was expressed as percentage relative standard deviation (% RSD) of peak area and accuracy was expressed as a percent of the nominal concentration. The summarized results of precision and accuracy are shown in Table-3. Low % RSD and good percent assay values proved the precision and accuracy of the method, respectively.

TABLE-3
PRECISION AND ACCURACY PARAMETERS FOR THE DETERMINATION OF GLYCOPYRROLATE AND FORMOTEROL FUMARATE BY THE PROPOSED METHOD

Injection No.	Formoterol fumarate		Glycopyrrolate	
	Peak area	Assay (%)	Peak area	Assay (%)
1	1295903	99.65	3168753	100.14
2	1299357	99.91	3164073	99.99
3	1292930	99.42	3167726	100.10
4	1299680	99.94	3161951	99.92
5	1298802	99.87	3169839	100.17
6	1293223	99.44	3160406	99.87
Mean	1296649	99.71	3165458	100.03
RSD (%)	0.237	0.237	0.122	0.123

Recovery study: The validity of the proposed method was assessed through recovery study by applying the standard addition technique. For this, standard glycopyrrolate and formoterol fumarate were spiked to placebo at three different concentration levels (50, 100 and 150 %). The mean percent recovery of drug at each level was determined. Results given in Table-4 showed that the suggested method is valid and applicable for the analysis of glycopyrrolate and formoterol fumarate with an acceptable percentage recovery. There was no interference from common excipients.

Robustness: Method robustness was investigated to find out whether small variations in chromatographic conditions such flow rate of mobile and column temperature affected system suitability for the analysis of glycopyrrolate and formoterol fumarate. Standard drug solution (glycopyrrolate 18.0 µg/mL and formoterol fumarate 9.6 µg/mL) was evaluated under test conditions. The system suitability parameters were determined (Table-5). From the results (Table-5), it was observed that small changes in the flow rate of mobile and column temperature had minimal effects on system suitability parameters. Hence the proposed method is robust.

Conclusion

An analytical method for the simultaneous estimation of glycopyrrolate and formoterol fumarate based on RP-HPLC technique with photodiode array detector was developed. The developed method has done with the necessary validation procedures, following ICH guidelines, for reliable analysis of glycopyrrolate and formoterol fumarate with adequate sensitivity, precision and accuracy for the routine analysis. Also the method proved to have suitable selectivity and robustness for the analysis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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TABLE-4
DETERMINATION OF GLYCOPYRROLATE AND FORMOTEROL FUMARATE BY THE PROPOSED METHOD AND APPLICATION OF STANDARD ADDITION TECHNIQUE

Spiked level (%)	Formoterol fumarate				Glycopyrrolate			
	Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean (%)	Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean (%)
50	4.80	4.78	99.68		9.00	9.00	99.99	
50	4.80	4.79	99.73	99.73	9.00	9.04	100.45	100.22
50	4.80	4.79	99.79		9.00	9.02	100.23	
100	9.60	9.56	99.62		18.00	18.03	100.14	
100	9.60	9.55	99.52	99.68	18.00	17.98	99.89	99.97
100	9.60	9.59	99.89		18.00	17.98	99.87	
150	14.40	14.38	99.86		27.00	26.98	99.91	
150	14.40	14.33	99.52	99.62	27.00	27.00	100.02	100.00
150	14.40	14.32	99.48		27.00	27.02	100.06	

TABLE-5
EFFECT OF VARIABLES ON THE SYSTEM SUITABILITY PARAMETERS OF GLYCOPYRROLATE AND FORMOTEROL FUMARATE

Parameter varied	Formoterol fumarate			Glycopyrrolate		
	Plate count	Tailing factor	Resolution	Plate count	Tailing factor	Resolution
Flow rate: 0.9 mL/min	6425	1.39	–	7637	1.39	3.51
Flow rate: 1.1 mL/min	7625	1.57	–	8477	1.53	3.65
Column temperature: 23 °C	6117	1.54	–	7384	1.52	3.45
Column temperature: 27 °C	7416	1.58	–	8419	1.53	3.69

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