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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ACLIDINIUM BROMIDE AND FORMOTEROL FUMARATE IN BULK AND INHALER FORMULATION

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ABSTRACT

Objective: To develop a simple, accurate, precise, rapid and sensitive method for the simultaneous estimation of Formoterol fumarate and Aclidinium bromide in pharmaceutical dosage form.

Methods: The chromatogram was run through Ascentis C_{18} 150 x 4.6 mm, 5µ. Mobile phase containing Water: Acetonitrile taken in the ratio 60:40 was pumped through the column at a flow rate of 1.0 ml/min. The temperature was maintained at 30 °C. The optimized wavelength selected was 220 nm.

Results: The retention times of Formoterol fumarate and Aclidinium bromide were found to be 2.953 min and 2.364 min. %RSD of the Aclidinium bromide and Formoterol fumarate was found to be 0.6 and 0.9, respectively. %Recovery was obtained as 99.81 % and 100.20% for Aclidinium bromide and Formoterol fumarate, respectively. LOD, LOQ values obtained from the Signal-to-noise ratio of Aclidinium bromide and Formoterol fumarate were 0.84 µg/ml, 2.56 µg/ml and 0.01 µg/ml, 0.03µg/ml respectively. Regression equation of Formoterol fumarate is y =9023x+268.67, and y = 4661.2x+1941.9 of Aclidinium bromide. Retention times were decreased and that run time was decreased, so the method developed was simple, rapid, sensitive and economical that can be adopted in regular quality control tests in Industries.

Conclusion: Developed and Validated Formoterol fumarate and Aclidinium bromide in pharmaceutical dosage form by using RP-HPLC method.

Keywords: Reverse phase-high-performance liquid chromatography, Validation, Formoterol fumarate, Aclidinium bromide

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INTRODUCTION

Aclidinium bromide with the IUPAC name *[[(3R)-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octan-3-yl]2-hydroxy-2,2-dithiophen-2-ylacetate; bromide]* [1], is an anticholinergic drug used to control and prevent symptoms caused by chronic obstructive pulmonary diseases (COPD) like bronchitis and emphysema [2]. The structure of Aclidinium bromide is shown in fig. 1.

Formoterol fumarate with the IUPAC name *[((E)-but-2-enedioic acid; N-[2-hydroxy-5-[(1S)-1-hydroxy-2-[[(2S)-1-(4-methoxyphenyl)Propan-2-yl]amino]ethyl] phenyl] formamide]* [3], is a long-acting bronchodilator used as a long-term treatment to prevent or to decrease wheezing and trouble breathing caused by asthma or COPD. The structure of Formoterol fumarate is shown in fig. 2.

Both drugs work by relaxing the respiratory muscles [2, 4].

HPLC is an accurate and sensitive method used for the quantitative analysis of several drugs [5, 6]. Literature shows a few methods for simultaneous estimation of Aclidinium Bromide and Formoterol Fumarate [7-10]. The present study aims to develop and validate an economical and effective HPLC method for simultaneous determination with good linearity and sensitivity for both drugs, which could be used in quality control analysis.

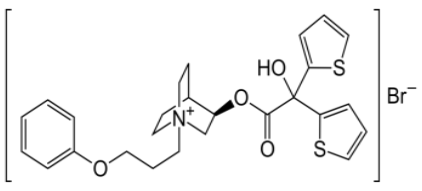

Fig. 1: Structure of aclidinium bromide [1]

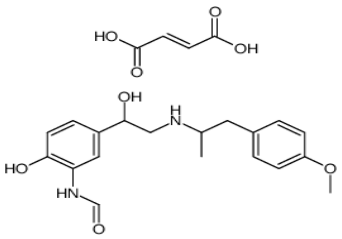

Fig. 2: Structure of formoterol fumarate [3]

MATERIALS AND METHODS

The API Aclidinium Bromide, Formoterol Fumarate was obtained from MSN Pharma Ltd, Hyderabad. The marketed formulation DuaklirPressair® (Formoterol fumarate and Aclidinium bromide inhaler), MSN Pharma Ltd, Hyderabad, India was used. Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen orthophosphate, Ortho-phosphoric acid are from Rankem. Denver Electronic balance, BVK Enterprise p^H meter and Ultrasonicator, Thermo Scientific Hot air oven and Refrigerator, Millipore BM2EA9672R, WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and autosampler integrated with Empower 2 Software. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Formoterol fumarate and Aclidinium bromide solutions.

Methodology

Diluent

Based upon the solubility of the drugs, Acetonitrile and Water taken in the ratio of 50:50 was selected as diluent.

Preparation of solutions

Preparation of standard stock solutions

Accurately weighed 3 mg of Formoterol fumarate, 100 mg of Aclidinium bromide and transferred individually to 50 ml volumetric flasks and 3/4th of diluents was added to these flasks and sonicated for 10 min. Flasks were make up with diluents and labelled as Standard stock solutions. (60µg/ml of Formoterol fumarate and 2000µg/ml of Aclidinium bromide).

Preparation of standard working solutions (100% solution)

1 ml from each stock solution was pipetted out and taken into a 10 ml volumetric flask and made up with a diluent. (6µg/ml of Formoterol fumarate and 200µg/ml of Aclidinium bromide).

Preparation of sample stock solution and sample working solution (100% solution)

The contents of the inhaler were collected by 50 actuations (1.2µg Formoterol fumarate and 40µg Aclidinium bromide) into a 50 ml volumetric flask. 20 ml acetonitrile was added and sonicated for 25 min and volume is made up to mark to yield 12 and 400µg. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVD).5 ml from this solution was pipetted out and taken into a 10 ml volumetric flask and made up with diluent. (6µg/ml of Formoterol fumarate and 200µg/ml of Aclidinium bromide).

Optimization of chromatographic conditions

Method development for the analysis of Aclidinium Bromide and Formoterol Fumarate was done by changing mobile phase ratios, buffers, flow rate, columns, and run time. Acceptable retention times, good resolution, tailing factor and theoretical plates were observed with optimized chromatographic conditions mentioned in table 1. The optimized chromatogram is shown in fig. 3. Validation and stability studies of the optimized method were performed according to the ICH guidelines [11].

Method validation

Validation was performed as per the ICH Q2B (R2) guidelines [11]. The method was validated for the parameters like system suitability, specificity, linearity, precision (system precision and repeatability), accuracy, the limit of detection and limit of quantification, robustness, and assay. Stability Studies like acid degradation, base degradation, oxidative degradation, thermal degradation, photostability degradation, and aqueous degradation were carried out as per ICH guidelines [12].

Table 1: Optimized chromatographic conditions

S. No.	Parameter	Condition
1	Mobile phase	60% Water: 40% Acetonitrile
2	Diluent	Water: Acetonitrile (50:50)
3	Column	Ascentis C_{18} (4.6 x 150 mm, 5 µm)
4	Wavelength	220 nm
5	Column temperature	30 °C
6	Injection volume	10 µl
7	Flow rate	1.0 ml/min
8	Run time	5 min
9	Retention time	2.364 min (Aclidinium Bromide) 2.953 min (Formoterol Fumarate)

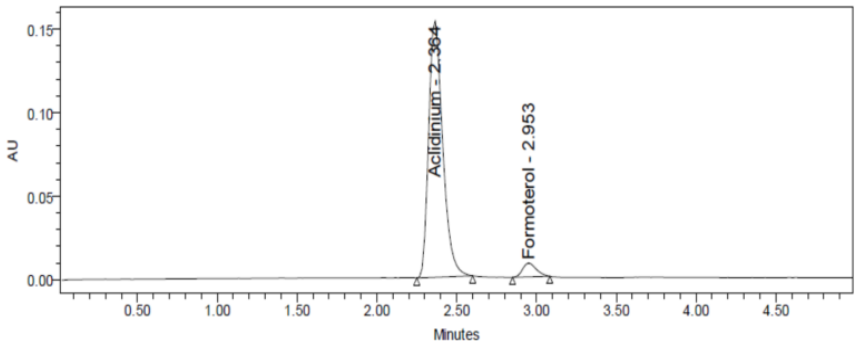

Fig. 3: Optimized chromatogram

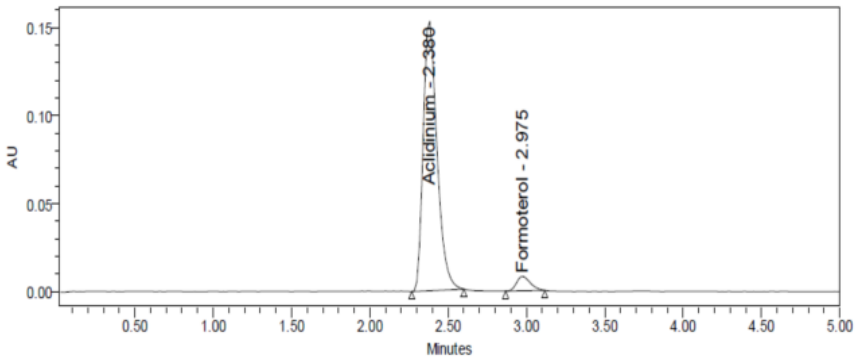

Fig. 4: Chromatogram for system suitability

System suitability

It is performed to verify that the analytical system is working properly and can give accurate and precise results. Standard solutions of Aclidinium Bromide (6 ppm) and Formoterol Fumarate (200 ppm) were injected six times and the parameters like resolution, peak tailing, and USP plate count were determined. The chromatogram was represented in fig. 4, and the results of system suitability were shown in table 2. According to ICH guidelines, plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitability parameters were passed and were within limits.

Table 2: System suitability parameters for Aclidinium bromide and Formoterol fumarate

S. No.	Aclidinium bromide			Formoterol fumarate			
Injection	Rt (min)	USP plate count	Tailing	Rt (min)	USP plate count	Tailing	Resolution
1	2.364	3518	1.31	2.953	5426	1.16	3.6
2	2.380	3554	1.33	2.975	5235	1.23	3.6
3	2.389	3461	1.34	2.993	5109	1.24	3.6
4	2.393	3467	1.37	3.000	5295	1.17	3.6
5	2.398	3574	1.33	3.000	5537	1.18	3.6
6	2.405	3580	1.31	3.009	5297	1.21	3.6

Specificity

The specificity of the method is performed by separately injecting the blank and placebo at sample solutions. The interference observed (if any) at the retention times of each analyte in all the chromatograms is evaluated. Chromatograms were as shown in fig. 5, fig. 6 and fig. 7. Retention times of Aclidinium Bromide and Formoterol Fumarate were 2.364 min and 2.953 min, respectively. The method is specified as no interfering peaks were observed in blank and place boat retention times of the drugs.

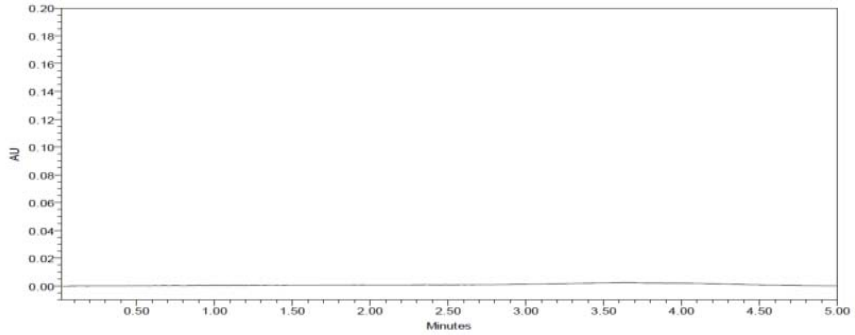

Fig. 5: Chromatogram of blank

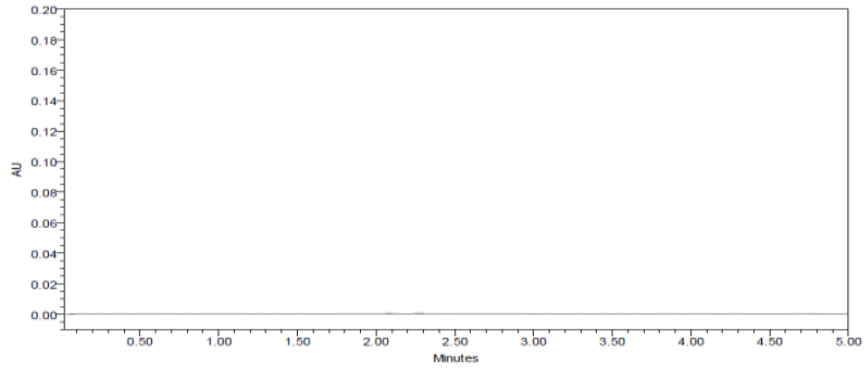

Fig. 6: Chromatogram of placebo

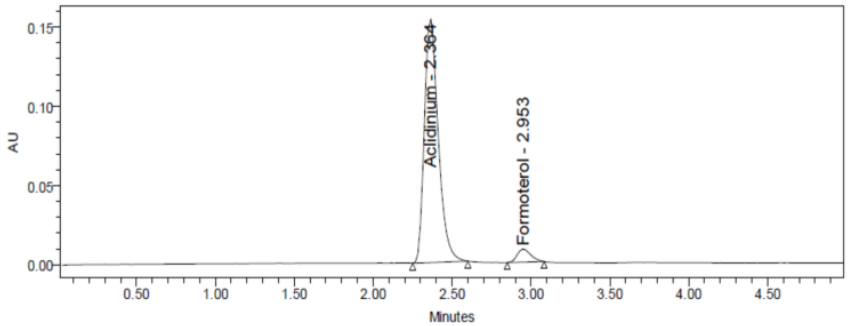

Fig. 7: Typical chromatogram

Linearity

Standard solutions of 25%, 50%, 75%, 100%, 125%, and 150% concentrations were prepared by taking 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 ml each from two standard stock solutions and make up to 10 ml. Six linear concentrations of Formoterol fumarate (1.5-9.0µg/ml) and Aclidinium bromide (50-300µg/ml) were injected in a duplicate manner. Peak areas were recorded for each injected concentration and the calibration curves-concentration vs. peak area were constructed fig. 8 and fig. 9. The results were given in table 3 and table 4. Linearity equations obtained for Formoterol fumarate was y = 9023x+268.67and of Aclidinium bromide was y =4661.2x+1941.9. Correlation coefficient obtained was 0.999.

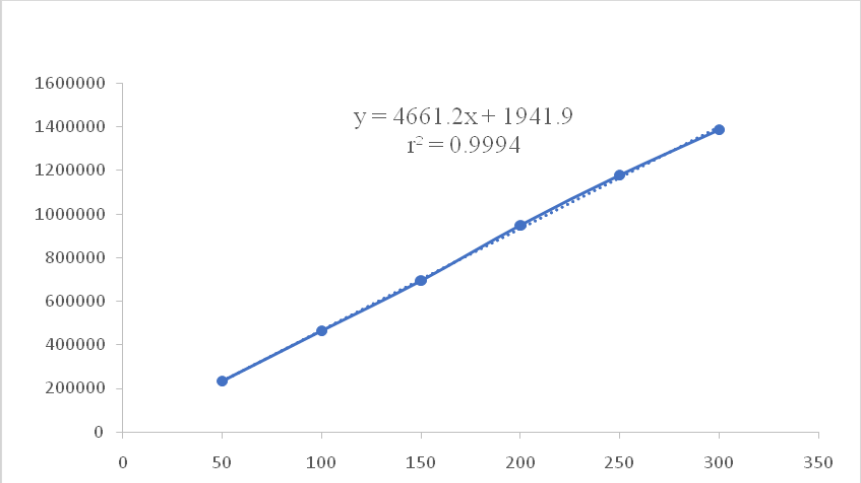

Fig. 8: Calibration curve of Aclidinium bromide

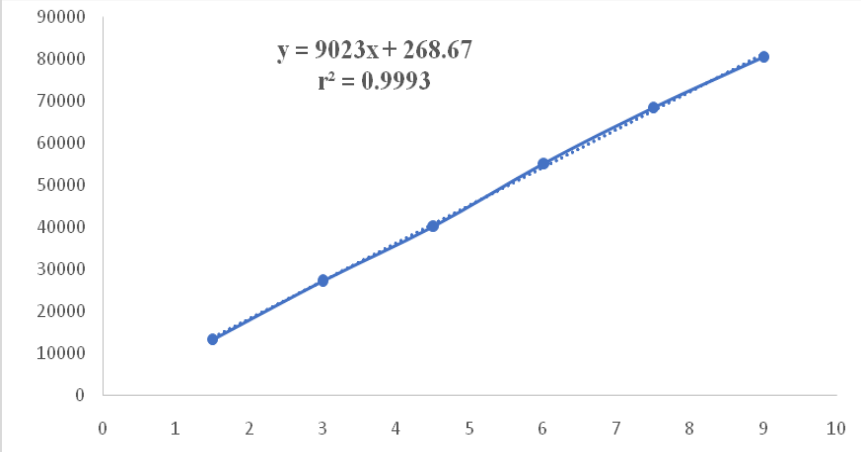

Fig. 9: Calibration curve of Formoterol fumarate

Table 3: Results for linearity of Aclidinium bromide

Aclidinium bromide	
Conc. (µg/ml)	**Peak area**
50	233919
100	464647
150	694073
200	948755
250	1178371
300	1386179

Table 4: Results for linearity of Formoterol fumarate

Formoterol fumarate	
Conc. (µg/ml)	**Peak area**
1.5	13531
3	27459
4.5	40365
6	55279
7.5	68596
9	80608

Precision

System precision

System precision was determined by injecting 15 µl standard solution six times and the chromatograms were recorded. Average area, standard deviation, and %RSD were calculated for two drugs and the results are shown in table 5. %RSD was obtained as 0.6% and 0.1%, respectively for Aclidinium Bromide and Formoterol Fumarate. As the limit of precision was less than 2, the method is precise.

Table 5: Results for system precision of Aclidinium bromide and Formoterol fumarate

S. No.	System precision	
	Area of aclidinium bromide	Area of formoterol fumarate
1.	935674	49682
2.	934514	50660
3.	947330	50449
4.	943197	50965
5.	944125	50702
6.	944718	50360
Mean	941593	496031
SD	5230.9	440.1
%RSD	0.6	0.1

Repeatability

Repeatability (Method precision) was determined by multiple sampling from a sample stock solution and six working sample solutions of the same concentrations were prepared, 15 µl injection from each working sample solution was given, and obtained areas were mentioned in table 6. Average area, standard deviation, and % RSD were calculated for two drugs and obtained as 0.3% and 0.8%, respectively for Aclidinium Bromide and Formoterol Fumarate.

As the limit of Precision was less than 2 the method is repeatable.

Table 6: Results for repeatability of Aclidinium bromide and Formoterol fumarate

Repeatability		
S. No.	Area of aclidinium bromide	Area of formoterol fumarate
1.	934899	50012
2.	930938	50196
3.	935516	50566
4.	938062	50747
5.	936682	50391
6.	935234	49663
Mean	935222	50263
SD	2397.5	392.4
%RSD	0.3	0.8

Accuracy

Three levels (50%, 100%, 150%) of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy. The results were shown in table 7 and table 8. Mean % Recovery was obtained as 99.83% and 100.20% for Aclidinium Bromide and Formoterol Fumarate, respectively.

Table 7: Accuracy results for Aclidinium bromide

% Level	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	Mean % recovery
50%	100	100.56	100.56	99.96%
	100	100.04	100.04	
	100	99.26	99.29	
100%	200	199.58	99.79	99.85%
	200	200.45	100.23	
	200	199.07	99.54	
150%	300	297.41	99.14	99.69%
	300	295.81	98.60	
	300	304.02	101.34	

Table 8: Accuracy results for Formoterol fumarate

% Level	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	Mean % recovery
50%	3	3.01	100.39	100.41%
	3	3.05	101.66	
	3	2.98	99.20	
100%	6	6.00	100.06	100.06%
	6	6.00	100.07	
	6	6.00	100.05	
150%	9	8.91	99.00	100.12%
	9	9.14	101.51	
	9	8.99	99.87	

Limit of detection and limit of quantification

The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. "LOD and LOQ is related to both the signal and the noise of the system and is usually defined as a peak whose signal-to-noise (S/N) ratio is at least 3:1 for LOD and 10:1 for LOQ" [13]. The results were shown in table 9.

Table 9: Results for LOD and LOQ of Aclidinium bromide and Formoterol fumarate

Drug	LOD (µg/ml)	LOQ (µg/ml)
Aclidinium Bromide	0.84	2.56
Formoterol Fumarate	0.01	0.03

Robustness

Robustness conditions like Flow rate minus (0.9 ml/min), Flow rate plus (1.1 ml/min), mobile phase minus (65W: 35A), mobile phase plus (55W: 45A), temperature minus (25 °C), and temperature plus (35 °C) were maintained and samples were injected in a duplicate manner. Results were given in table 10. System suitability parameters were not much affected and %RSD was within the limit. Hence the method was considered to be robust.

Assay

Assay was performed with (DuaklirPressair®) bearing the label claim Formoterol 12 µg, Aclidinium 400 µg. 20 µl of the Standard and Sample solutions were injected into Chromatographic System and areas for Aclidinium Bromide and Formoterol Fumarate were measured and results were shown in table 11. The average % Assay for Formoterol fumarate and Aclidinium bromide obtained was 99.39% and 99.12%, respectively.

Stability studies

Acid degradation studies

1 ml 2N Hydrochloric acid was added to 1 ml stock solution of Aclidinium Bromide and Formoterol Fumarate, refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 6µg/ml and 200µg/ml solution and 10 µl solution was injected into the system to assess the stability of the sample. The results were given in table 12.

Table 10: Results for the robustness of Aclidinium bromide and Formoterol fumarate

S. No.	Condition	%RSD of Aclidinium bromide	%RSD of formoterol fumarate
1	Flow rate (-) 0.90 ml/min	0.9	0.9
2	Flow rate (+) 1.1 ml/min	0.5	0.3
3	Mobile phase (-) 65W: 35A	0.5	1.1
4	Mobile phase (+) 55W: 45A	0.5	0.7
5	Temperature (-) 25 °C	0.3	0.1
6	Temperature (+) 35 °C	0.7	0.2

Table 11: Results for assay of Aclidinium bromide and Formoterol fumarate

S. No.	Aclidinium bromide			Formoterol fumarate		
	Standard area	Sample area	% Assay	Standard area	Sample area	% Assay
1	935674	934899	99.09	49682	50012	98.89
2	934514	930938	98.67	50660	50196	99.26
3	947330	935516	99.16	50449	50566	99.99
4	943197	938062	99.43	50965	50747	100.35
5	944125	936682	99.28	50702	50391	99.64
6	944718	935234	99.13	50360	49663	98.20
Avg	941593	935222	99.12	50366	50263	99.39
SD	5230.9	2397.5	0.25	440.1	392.4	0.8
%RSD	0.6	0.3	0.3	0.9	0.8	0.8

Alkali degradation studies

1 ml 2N NaOH was added to 1 ml stock solution of Aclidinium Bromide and Formoterol Fumarate and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 6µg/ml and 200µg/ml solution and 10 µl solution was injected into the system to assess the stability of the sample. The results were given in table 12.

Oxidative degradation studies

1 ml 20% H_2O_2 was added to 1 ml of stock solution of Aclidinium Bromide and Formoterol Fumarate. The solutions were kept for 30 min at 60 °C. The resultant solution was diluted to obtain 6µg/ml and 200µg/ml solution and 10 µl were injected into the system to assess the stability of the sample. The results were given in table 12.

Thermal degradation studies

The standard drug solution was placed in an oven at 105 °C for 1 h to study dry heat degradation. The resultant solution was diluted to 6µg/ml and 200µg/ml solution and 10 µl were injected into the system to assess the stability of the sample. The results were given in table 12.

Photostability studies

The photochemical stability of the drug was studied by exposing 60µg/ml Aclidinium Bromide and 2000µg/ml Formoterol Fumarate solution to UV light by keeping the beaker in UV Chamber for one day. The resultant solution was diluted to obtain 6µg/ml and 200µg/ml solution and 10 µl were injected into the system to assess the stability of the sample. The results were given in table 12.

Table 12: Degradation data of Aclidinium bromide and Formoterol fumarate

S. No.	Degradation condition	Aclidinium bromide			Formoterol fumarate		
		Area	% Recover	% Drug degraded	Area	% Recover	%Drug degraded
1	Acid	841207	89.16	10.84	45312	89.60	10.40
2	Alkali	922161	97.74	2.26	49724	98.33	1.67
3	Oxidation	814730	86.35	13.65	48637	96.18	3.82
4	Thermal	923257	97.86	2.14	49260	97.41	2.59
5	UV	926891	98.24	1.76	50002	98.88	1.12
6	Water	940657	98.24	1.76	50312	99.49	0.51

Aqueous degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 1h at 60 °C. The resultant solution was diluted to 6µg/ml and 200µg/ml and 10 µl were injected into the system to assess the stability of the sample. The results were given in table 12.

CONCLUSION

In the present study, an attempt was made to develop a simple, accurate, precise, rapid and sensitive method was developed for the simultaneous estimation of the Aclidinium bromide and Formoterol fumarate in bulk and dosage form. Retention time of Aclidinium and Formoterol was found to be 2.364 min and 2.953 min. %RSD of the Aclidinium and Formoterol were and found to be 0.6 and 0.9, respectively. % Recovery was obtained as 99.69%-99.96% and 100.12%-100.41% for Aclidinium bromide and Formoterol fumarate, respectively. LOD, LOQ values obtained from Signal-to-noise ratio of Aclidinium bromide and Formoterol fumarate were 0.8, 2.56, and 0.01, 0.03, respectively. Regression equation of Formoterol fumarate is y =9023x+268.67, and y = 4661.2x+1941.9 of Aclidinium bromide. Retention times were decreased, and that run time was decreased, so the method developed was simple and economical, which is useful in pharmaceutical industries.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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