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Simultaneous Estimation Of Aclidinium Bromide And Formoterol Fumarate In Combined Formulation By Rp-Hplc Method

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Abstract

A simple, Accurate, precise method was developed for the simultaneous estimation of the Formoterol and Aclidinium in Pharmacuetical dosage form. Chromatogram was run through Kromosil C18 250 x 4.6 mm, 5µ. Mobile phase containing Buffer 0.1% OPA: Acetonitrile taken in the ratio 55:45 was pumped through column at a flow rate of 0.8ml/min. Buffer used in this method was 0.1% OPA buffer. Retention time of Formoterol and Aclidinium were found to be 2.921 min and 2.402 min. %RSD of the Aclidinium and Formoterol were and found to be 0.5 and 0.4 respectively. %Recovery was obtained as 99.72 % and 99.71% for Aclidinium and Formoterol respectively. LOD, LOQ values obtained from regression equations of Aclidinium and Formoterol were 0.05, 0.16 and 1.40, 4.23 respectively. Regression equation of Formoterol is y = 69552x + 10314, and y = 41057x + 71071 of Aclidinium. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Key Words: Formoterol, Aclidinium, RP-HPLC

INTRODUCTION:

Aclidinium[1] is a long-acting, reversible antagonist at muscarinic receptors, with equal affinity to all five subtypes, but with a half-life dissociation of 29.2 hours from subtype M3, or six times longer than that from M2. Inhaled Formoterol works like other β2 agonists, which causes bronchodilation by relaxing the smooth muscle in the airway to treat asthma exacerbation. A literature review resulted some methods of analysis in inhalation and human serum by volatmmetry[2], in urine by gas chromatography mass spectrometry[3], UV spectroscopy[4,5] for the estimation of formoterol either alone and in other combinations[6,7,8,9,10] and chromatographic methods were also developed for the determination of aclidinium and formoterol in their dosage form[11,12].

The main aim of the project work is to develop a novel RP-HPLC method which is able to separate and quantify the drug candidates selected for study viz., Aclidinium bromide and Formoterol fumarate present in its pure form as well as formulation and validate the method by ICH Q2 (R1)[13] guidelines with demonstrable accuracy, linearity, precision and robustness.

MATERIALS AND METHODS

Materials:

- Formoterol and Aclidinium pure drugs (API), Combination Formoterol fumarate and Aclidinium bromide inhaler (Duaklir®), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem

Instruments:

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises
- WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software.
- UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Formoterol and Aclidinium solutions.

Methods:

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions: Accurately weighed 3mg of Formoterol, 100mg of Aclidinium and transferred to 50ml volumetric flask and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (60µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (6µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of Sample solutions: The contents of nasal spray deliveried by 50 actuations (1.2&40 mcg each) were collected in 50 ml volumetric flask. Then 20ml acetonitrile was added , sonicated for 25 min and made up to mark to yield 12&400µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF)
5ml from sample stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (6µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of buffer:

0.1%OPA Buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Validation:

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Formoterol (6ppm) and Aclidinium (200ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Standard stock solutions: Accurately weighed 3mg of Formoterol, 100mg of Aclidinium and transferred to 50ml volumetric flask and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (60µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (6µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of Sample solutions: The contents of nasal spray deliveried by 50 actuations (1.2&40 mcg each) were collected in 10 ml volumetric flask. Then 8ml acetonitrile was added , sonicated for 25 min and made up to mark to yield 12&400 µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF)

5ml from sample stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (6µg/ml of Formoterol and 200µg/ml of Aclidinium)

Linearity:

Preparation of Standard stock solutions: Accurately weighed 3mg of Formoterol, 100mg of Aclidinium and transferred to 50ml volumetric flask and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (60µg/ml of Formoterol and 200µg/ml of Aclidinium)

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (1.5µg/ml of Formoterol and 50µg/ml of Aclidinium)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (3µg/ml of Formoterol and 100µg/ml of Aclidinium)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (4.5µg/ml of Formoterol and 150µg/ml of Aclidinium)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (6.0µg/ml of Formoterol and 200µg/ml of Aclidinium)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (7.5µg/ml of Formoterol and 250µg/ml of Aclidinium)

150% Standard solution: 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (9.0µg/ml of Formoterol and 300µg/ml of Aclidinium)

Accuracy:

Preparation of Standard stock solutions: Accurately weighed 3mg of Formoterol, 100mg of Aclidinium and transferred to 50ml volumetric flask and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (60µg/ml of Formoterol and 200µg/ml of Aclidinium)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102
Robustness: Small delibe rate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (0.7ml/min), Flow plus (0.9ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Formoterol, Aclidinium, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Formoterol, Aclidinium, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:

Oxidation:

To 1 ml of stock solution of Formoterol and Aclidinium, 1 ml of 20% hydrogen peroxide (H2O2)was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, there sultant solution was diluted to obtain 6µg/ml&200µg/ml solutionand 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stocks solution Formoterol and Aclidinium, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c.The resultant solution was diluted to obtain 6µg/ml&200µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Formoterol and Aclidinium, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The result ant solution was diluted to obtain 6µg/ml&200µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was place dinoven at 105°C for1h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 6µg/ml&200µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 60µg/ml&2000µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 6µg/ml&200µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxingthedruginwaterfor1hrs atatemperature of 60º. For HPLC study, the resultant solution was diluted to 6µg/ml&200µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

RESULTS AND DISCUSSION

Optimized wavelength selected was 250nm.

Method development: Method development was done by changing various, mobile phase ratios, buffers etc.

Optimized method:

Chromatographic conditions:

Mobile phase **:** 55% OPA: 45% 0.1% OPA

Flow rate **:** 0.8ml/min

Column **:** Kromosil C18 (4.6 x 250mm, 5µm)

Detector wave length **:** 250nm

Column temperature **:** 30°C

Injection volume **:** 10µL

Run time **:** 5 min

Diluent **:** Water and Acetonitrile in the ratio 50:50

Results : In this trail by using same column but changing the mobile phase ratio andboth peaks have good resolution, tailing Factor, theoretical plate count and resolution.

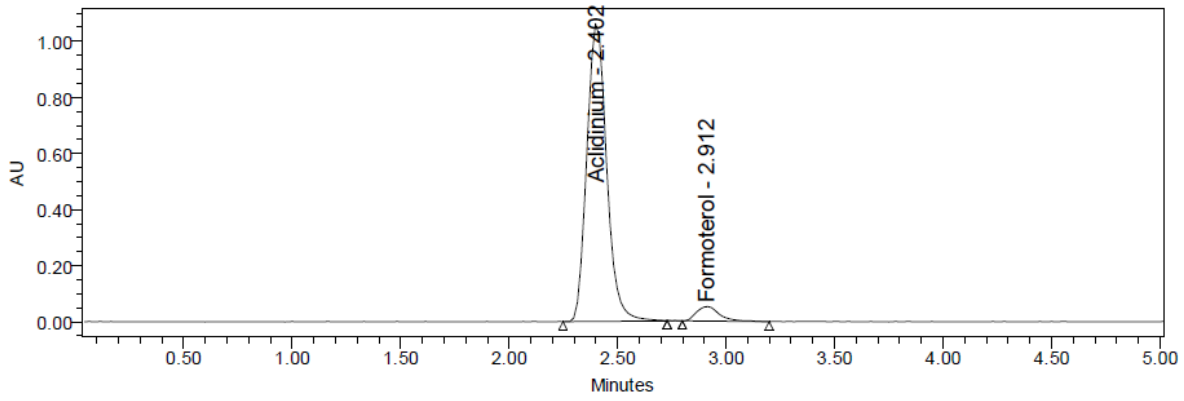

Fig 6.3 Optimized chromatogram

Observation: Formoterol and Aclidinium were eluted at 2.921 min and 2.402 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table:6.1 System suitability parameters for Formoterol and Aclidinium

S no	Aclidinium			Formoterol			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.396	3354	1.15	2.904	4092	1.45	2.9
2	2.402	3410	1.16	2.912	4087	1.51	2.9
3	2.404	3601	1.16	2.912	4315	1.37	2.9
4	2.406	3226	1.17	2.914	3840	1.49	2.8
5	2.406	3228	1.16	2.924	3861	1.4	2.9
6	2.407	3323	1.15	2.928	4000	1.42	2.9

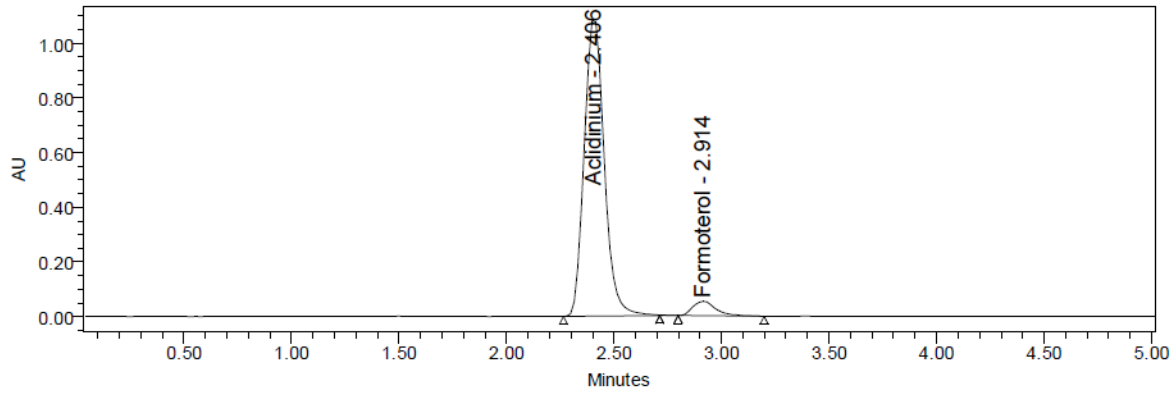

Fig 6.9 System suitability Chromatogram

Discussion: 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 suitable parameters were passed and were within the lim

Validation:

Specificity:

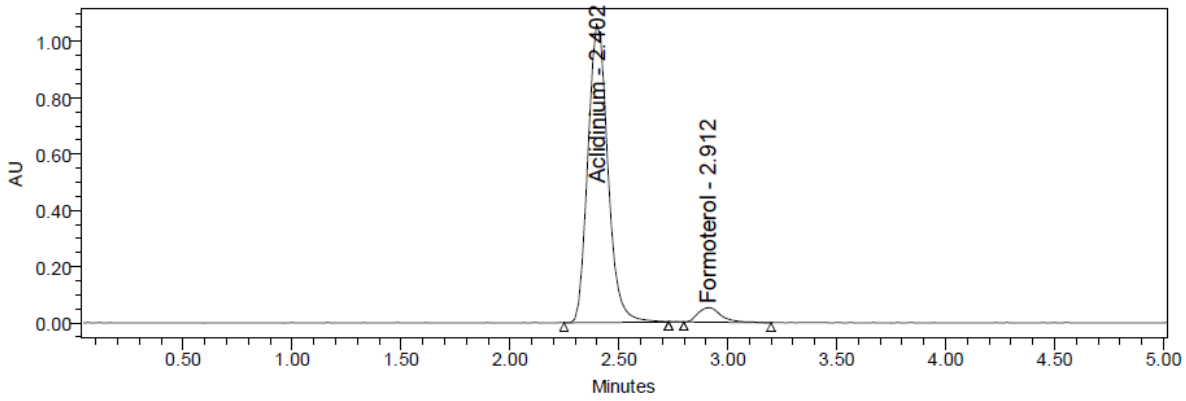

Fig 6.12 Typical Chromatogram

Discussion: Retention times of Aclidinium and Formoterol were 2.912min and 2.402 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity:

Table 6.2 Linearity table for Formoterol and Aclidinium.

Formoterol		**Aclidinium**	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
1.5	117395	50	2094106
3	227981	100	4253904
4.5	324676	150	6326972
6	431608	200	8175587
7.5	531949	250	10523680

9	629486	300	12232899

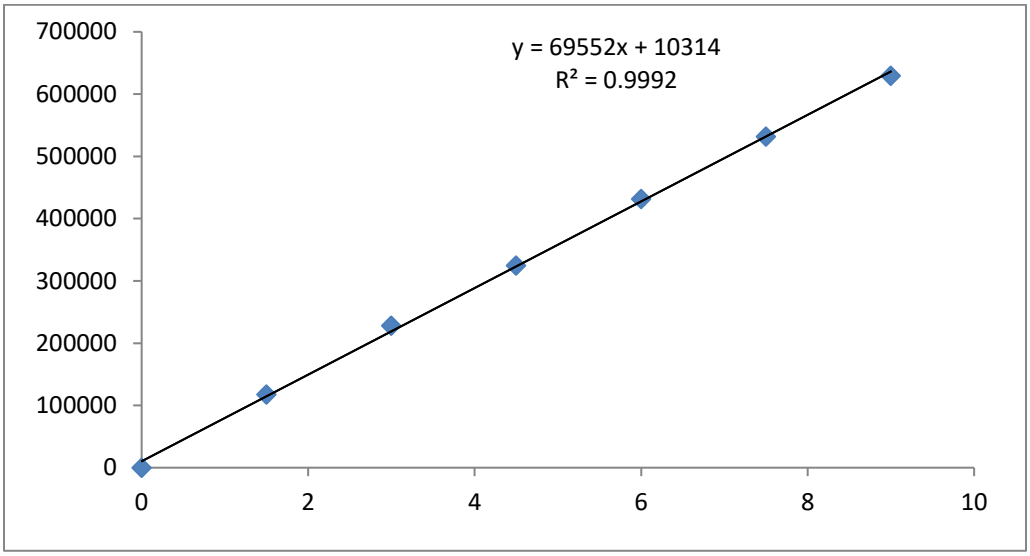

Fig No. 6.13 Calibration curve of Formoterol

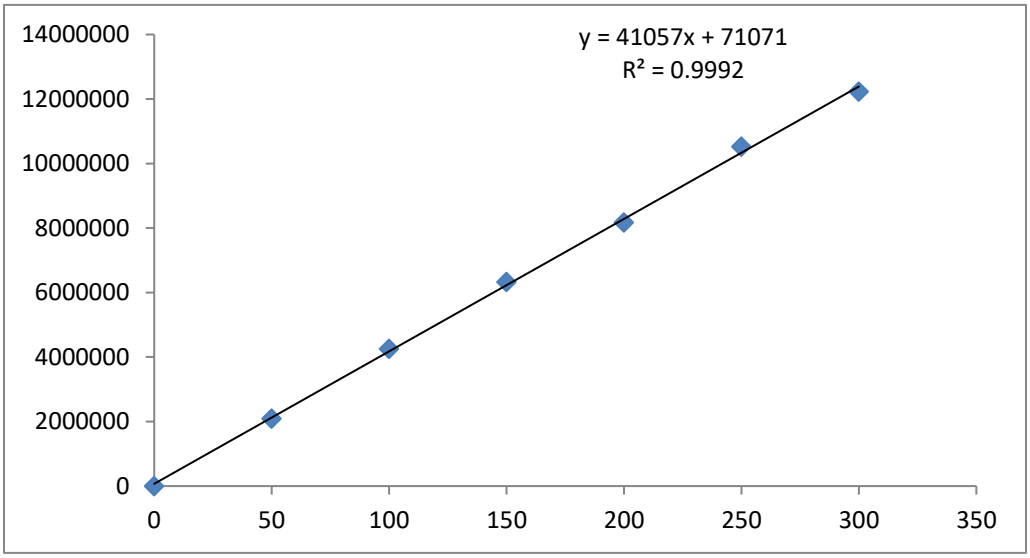

Fig No. 6.14 Calibration curve of Aclidinium

Discussion: Six linear concentrations of Formoterol (1.5-9.0µg/ml) and Aclidinium (50-300µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Formoterol was y = 69552x + 10314 and of Aclidinium was y = 41057x + 71071.Correlation coefficient obtained was 0.999 for the two drugs.

Precision:

System Precision:

Table 6.3 System precision table of Formoterol and Aclidinium

S. No	Area of Formoterol	Area of Aclidinium
1.	430181	8147355
2.	429871	8145785
3.	430650	8104160
4.	431602	8093502
5.	434577	8155067
6.	428810	8179853
Mean	430949	8137620
S.D	2000.3	32605.0
%RSD	0.5	0.4

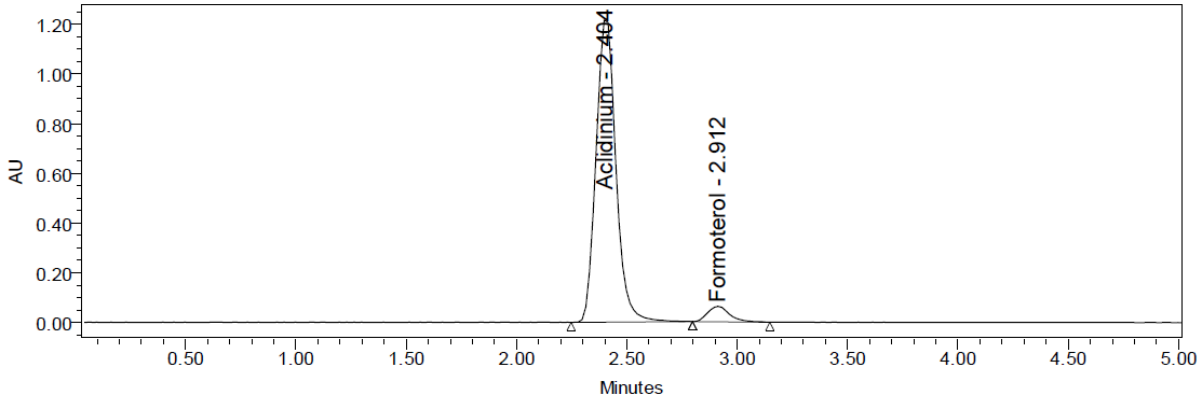

Fig 6.21 System precision chromatogram

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.5% and 0.4% respectively for Formoterol and Aclidinium .As the limit of Precision was less than “2” the system precision was passed in this method.

Repeatability:

Table 6.4 Repeatability table of Formoterol and Aclidinium

S. No	Area of Formoterol	Area of Aclidinium
1.	429137	8138486
2.	429799	8123724
3.	429569	8082289
4.	431301	8099998
5.	430023	8172690
6.	431248	8154565
Mean	430180	8128625
S.D	897.6	33771.0
%RSD	0.2	0.4

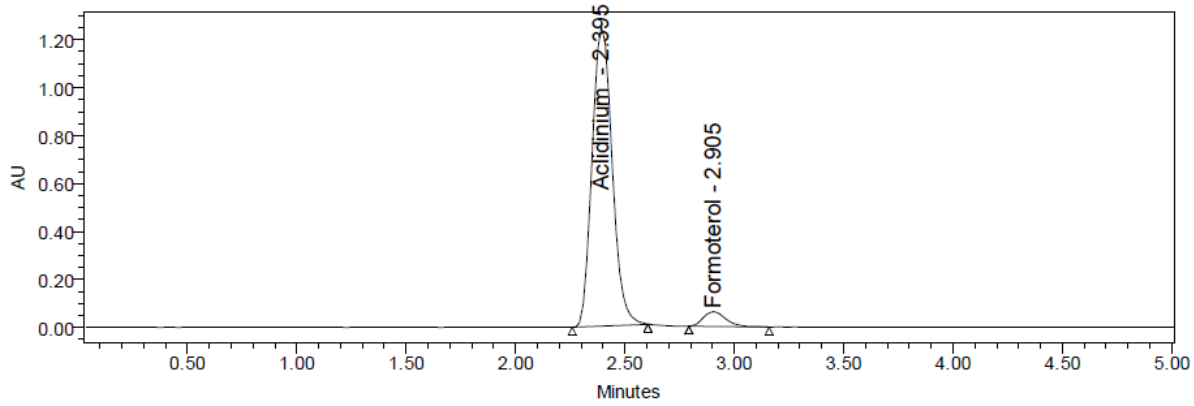

Fig No. 6.22 Repeatability chromatogram

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.2% and 0.4% respectively for Formoterol and Aclidinium. As the limit of Precision was less than "2" the system precision was passed in this method.

Intermediate precision (Day_ Day Precision):

Table 6.5 Intermediate precision table of Formoterol and Aclidinium

S. No	Area of Formoterol	Area of Aclidinium

1.	371927	7939225
2.	372172	7908131
3.	376711	7993679
4.	373098	7904411
5.	377434	7927197
6.	381440	7957459
Mean	375464	7938350
S.D	3744.2	33516.1
%RSD	1.0	0.4

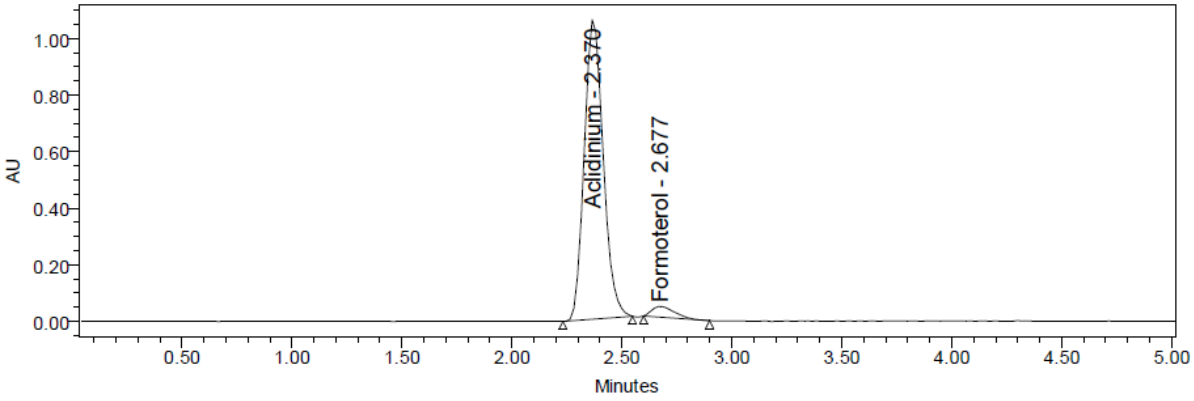

Fig: 6.23 Inter Day precision Chromatogram

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 1.0% and 0.4% respectively for Formoterol and Aclidinium. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Table 6.6 Accuracy table of Formoterol

% Level	**Amount Spiked (µg/mL)**	**Amount recovered**	**% Recovery**	Mean %Recovery

		(µg/mL)		
50%	3	2.99	99.67	99.72%
	3	3.03	101.10	
	3	2.99	99.69	
100%	6	5.96	99.35	
	6	5.99	99.83	
	6	5.99	99.80	
150%	9	8.93	99.18	
	9	8.93	99.24	
	9	8.96	99.60	

Table 6.7 Accuracy table of Aclidinium

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	100	99.99	99.99	99.71%
	100	99.81	99.81	
	100	100.46	100.46	
100%	200	201.93	100.97	
	200	197.90	98.95	

	200	200.10	100.05	
150%	300	297.26	99.09	
	300	296.64	98.88	
	300	297.72	99.24	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.72% and 99.71% for Formoterol and Aclidinium respectively.

Sensitivity:

Table 6.8 Sensitivity table of Formoterol and Aclidinium

Molecule	LOD	LOQ
Formoterol	0.05	0.16
Aclidinium	1.40	4.23

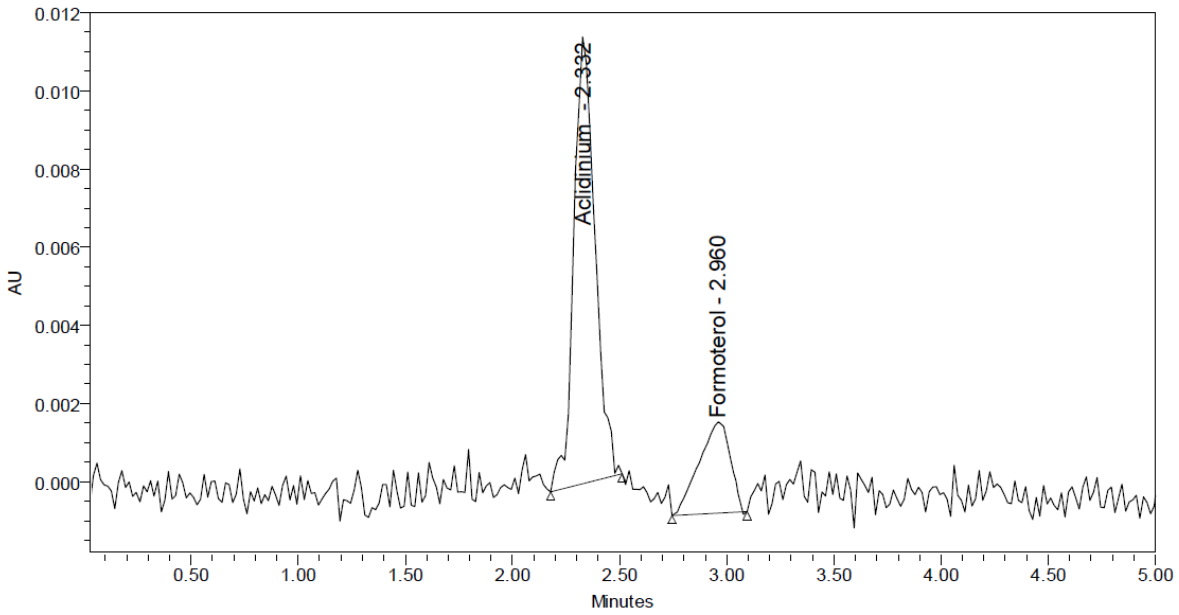

Fig. No. 6.27 LOD Chromatogram of Standard

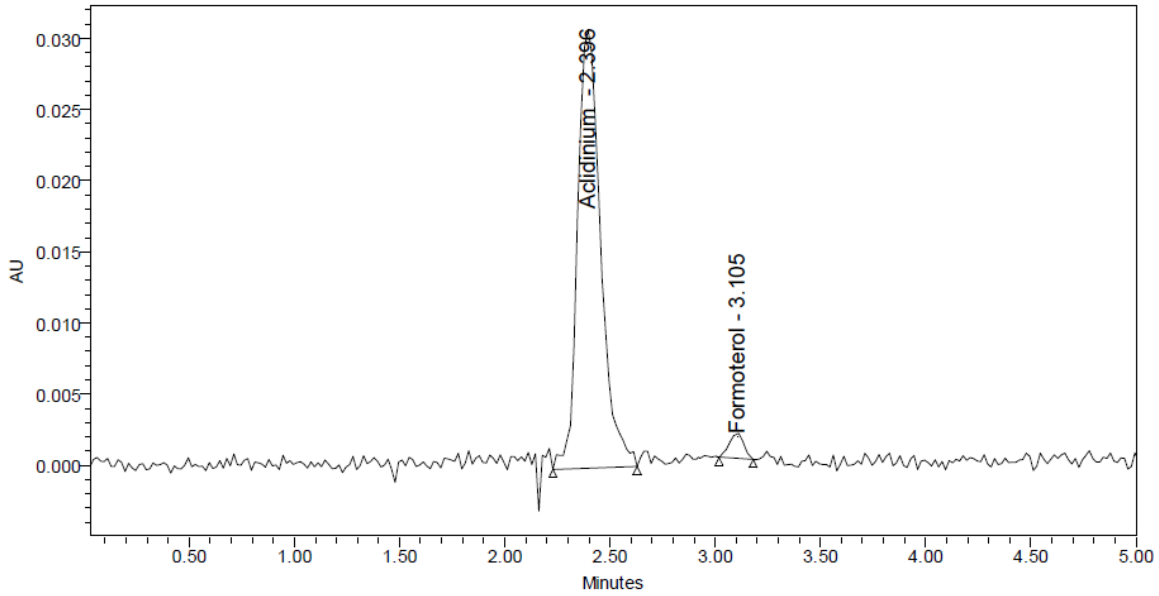

Fig.No. 6.28 LOQ Chromatogram of of Standard

Robustness:

Table 6.9 Robustness data for Formoterol and Aclidinium.

S.no	Condition	%RSD of Aclidinium	%RSD of Formoterol
1	Flow rate (-) 0.7ml/min	0.5	0.5
2	Flow rate (+) 0.9ml/min	0.5	0.4
3	Mobile phase (-) 60B:40A	0.2	0.2
4	Mobile phase (+) 50B:50A	1.3	1.1
5	Temperature (-) 25°C	0.4	0.7
6	Temperature (+) 35°C	0.7	0.8

Discussion: Robustness conditions like Flow minus (0.7ml/min), Flow plus (0.9ml/min), mobile phase minus (60B:40A), mobile phase plus (50B:50A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Formoterol and Aclidinium

Assay:,(Duaklir pressair)bearing the label claim Formoterol 12mcg, Aclidinium 400mcg. Assay was performed with the above formulation. Average % Assay for Formoterol and Aclidinium obtained was 99.62% and 99.69% respectively

Table 6.10 Assay Data of Formoterol

S.no	Standard Area	Sample area	% Assay
1	430181	429137	99.38
2	429871	429799	99.53
3	430650	429569	99.48
4	431602	431301	99.88
5	434577	430023	99.59
6	428810	431248	99.87
Avg	430564	430180	99.62
Stdev	2000.3	897.6	0.2
%RSD	0.5	0.2	0.2

Table 6.11 Assay Data of Aclidinium

S.no	Standard Area	Sample area	% Assay
1	8147355	8138486	99.81
2	8145785	8123724	99.63
3	8104160	8082289	99.12
4	8093502	8099998	99.34
5	8155067	8172690	100.23
6	8179853	8154565	100.01
Avg	8137620	8128625	99.69

Stdev	32605.0	33771.0	0.41
%RSD	0.4	0.4	0.4

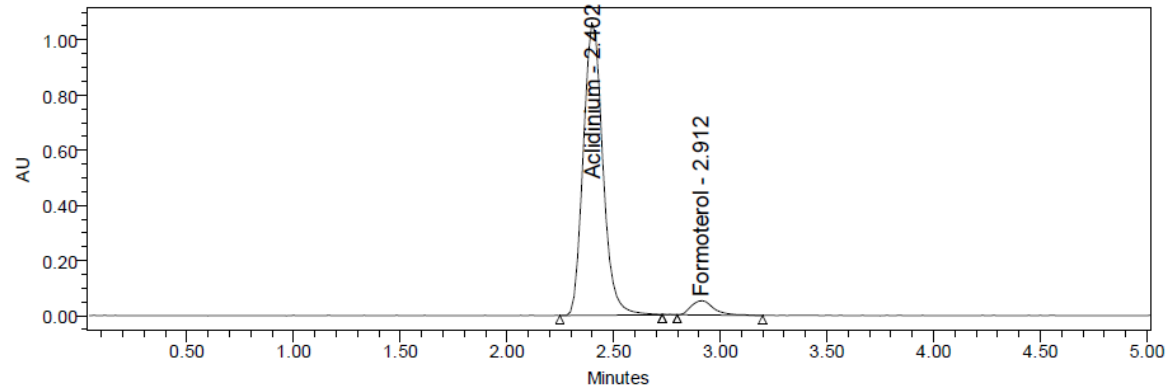

Fig 6.35 Chromatogram of working standard solution

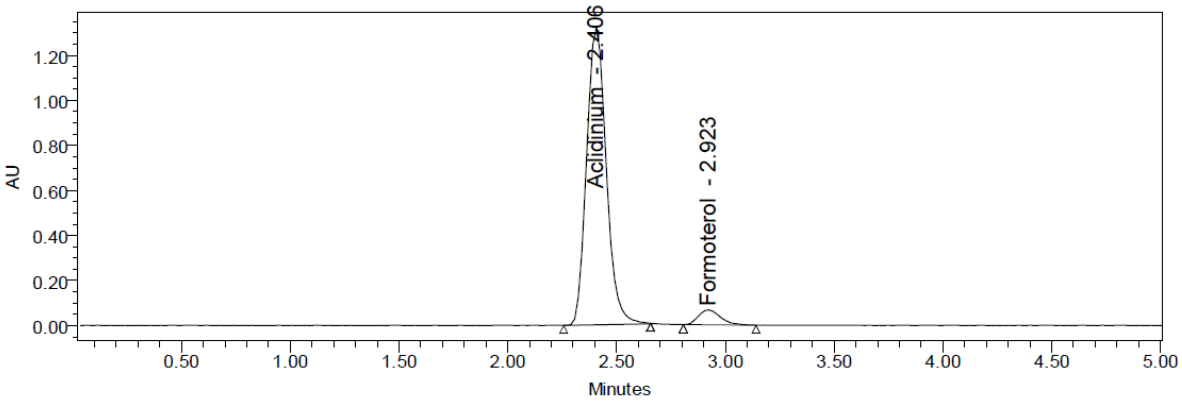

Fig No. 6.36 Chromatogram of working sample solution

6.8. Degradation data

Type of degradation	Formoterol			Aclidinium		
	AREA	%RECOVERED	% DEGRADED	AREA	%RECOVERED	% DEGRADED
Acid	400619	92.78	7.22	7708190	94.53	5.47
Base	406868	94.22	5.78	7720617	94.69	5.31
Peroxide	393603	91.15	8.85	7600892	93.22	6.78
Thermal	417731	96.74	3.26	7907451	96.98	3.02
Uv	420639	97.41	2.59	8044008	98.65	1.35

Water	431431	99.91	0.09	8096108	98.65	1.35

Degradation chromatograms

Acid degradation chromatogram

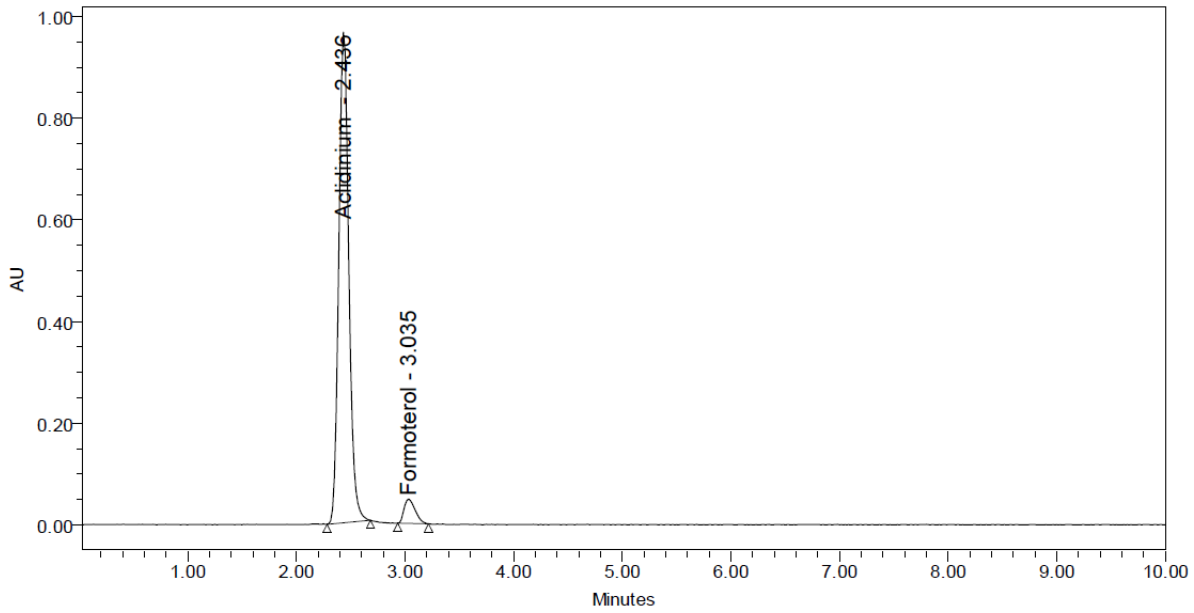

Fig.6.37 acid

Base degradation chromatogram

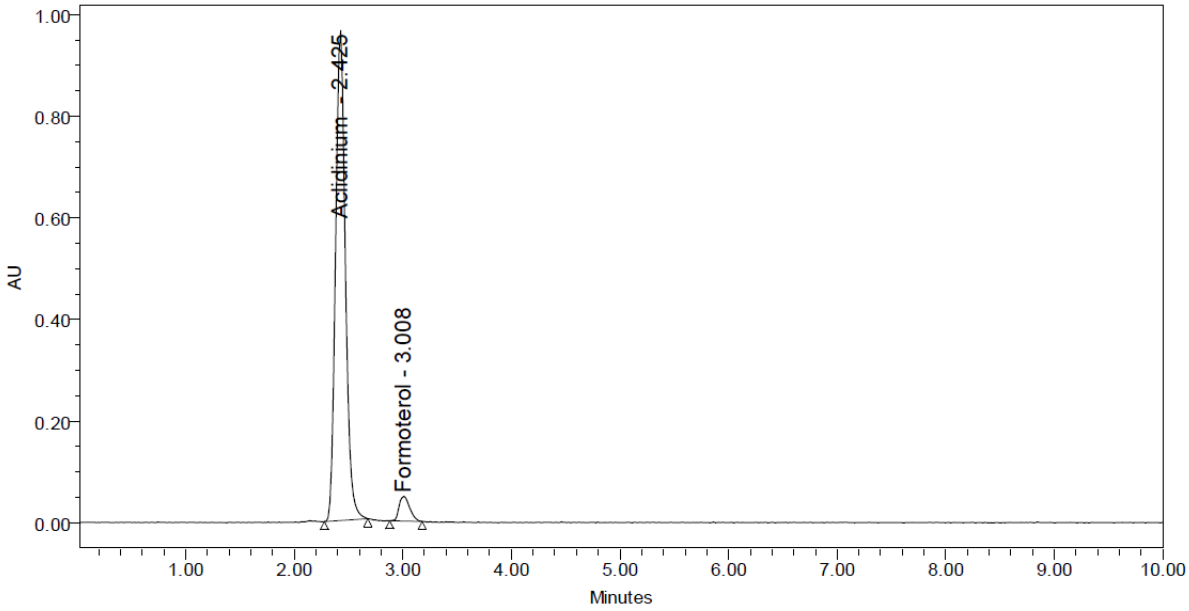

Fig.6.38 base

Peroxide degradation chromatogram

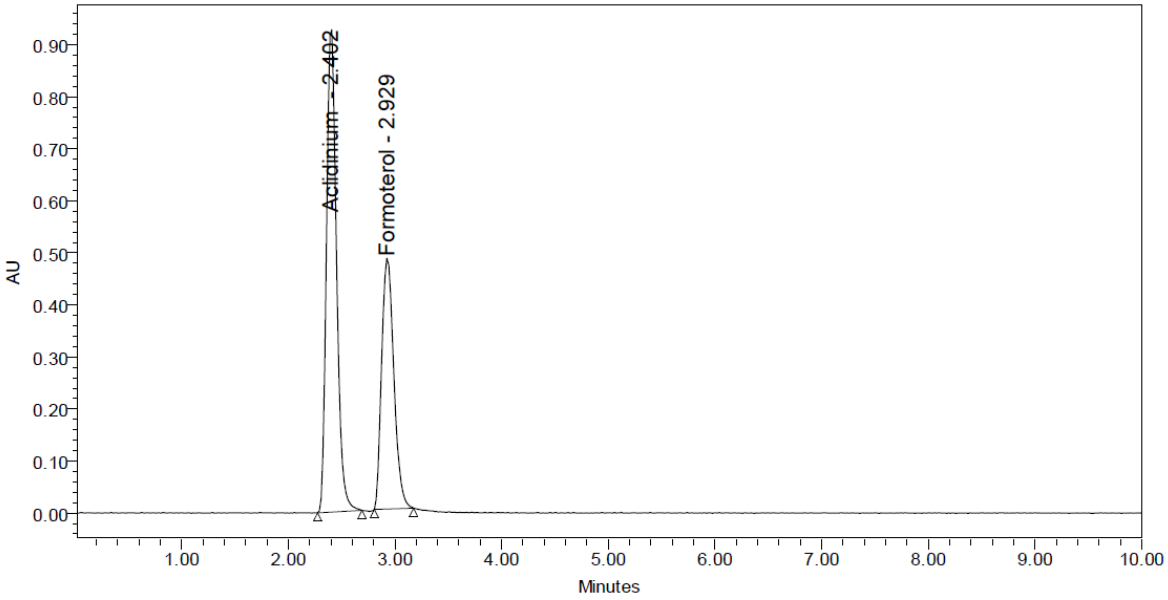

Fig.6.39 peroxide

Thermal degradation chromatogram

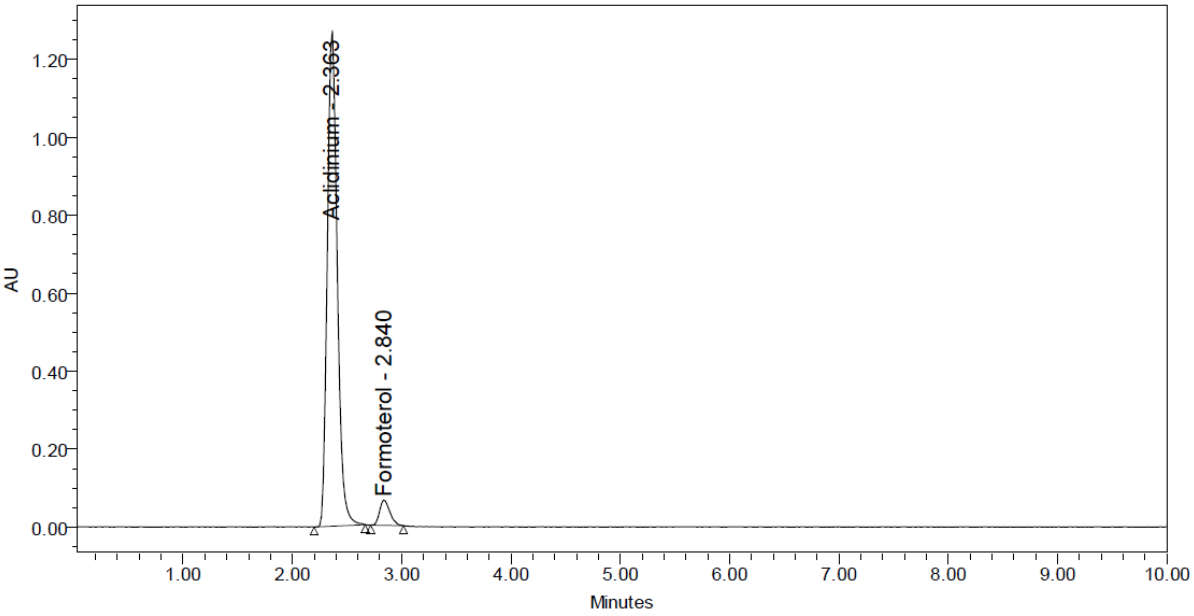

Fig.6.40 thermal

Uv degradation chromatogram

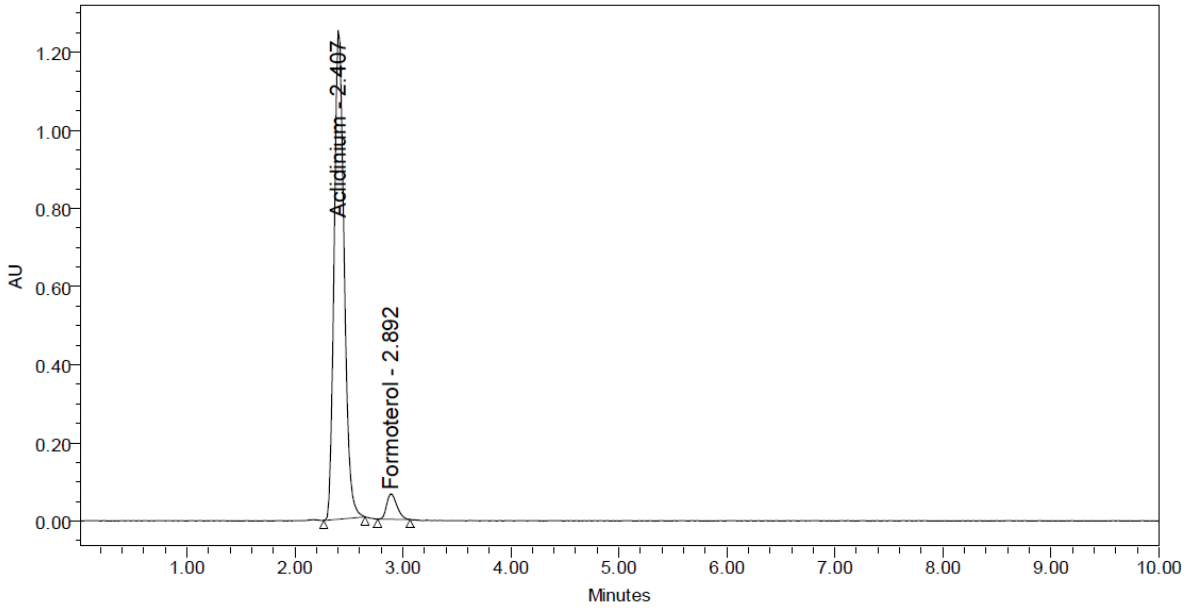

Fig.6.41 UV

Water degradation chromatogram

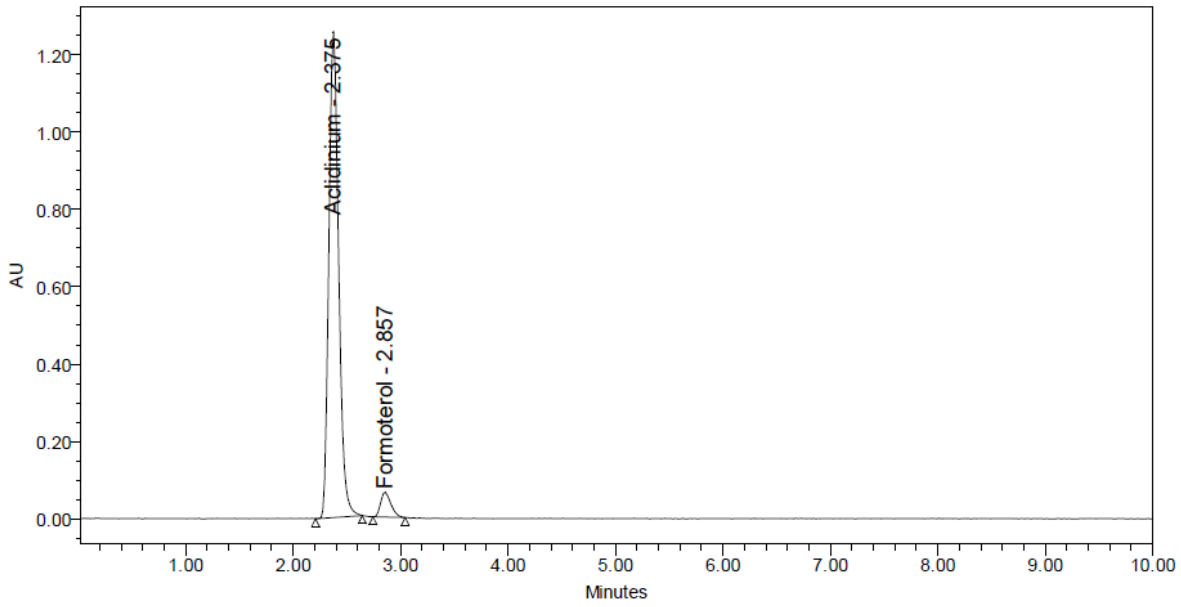

Fig.6.42 water

Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Aclidinium and Formoterol in bulk and dosage form. Retention time of Aclidinium and Formoterol were found to be 2.402 min and 2.912 min. %RSD of the Aclidinium and Formoterol were and found to be 0.4 and

0.5respectively. %Recovery was obtained as 100.41% and 100.57% for Aclidinium and Formoterol respectively. LOD, LOQ values obtained from regression equations of Aclidinium and Formoterol were 1.40, 4.23 and 0.05, 0.16 respectively. Regression equation of Formoterol is y = 69552x + 10314, and yy = 41057x + 71071 of Aclidinium. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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