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Research Article

Development and Validation of Stability Indicating Assay Method of Salbutamol Sulphate Metered Dose Inhaler by HPLC

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Abstract

In this study a simple, accurate, precise and sensitive high performance liquid chromatography (HPLC) method for the determination of Salbutamol and its degraded products in a Salbutamol sulphate inhaler have been developed and validated. The analysis was carried out using a Synergi 4µm Polar-RP 80A^0 150mm x 4.6mm column with a mobile phase consisting 75:25 of ammonium acetate buffer and methanol. The system suitability was assessed by analyzing standard solution containing Salbutamol. Syringe filter evaluation showed that 0.20 µm syringe filter is suitable for intended use. From the specificity and forced degradation study, it was observed that no peak from placebo, impurities and potential degradation was co-eluting at the retention time of Salbutamol and the assay result of spiked sample was unaffected by the presence of placebo and known impurities. The Salbutamol peak passed the peak purity testing. The proposed method was accurate with 99. 58% recovery for Salbutamol and precise (%RSD of area of assay repeatability, intermediate precision and reproducibility were found 0.65%, 0.99% and 0.61% respectively).From the linearity study the correlation coefficient was found 0.999, indicating that the method is linear over 0.240 ppm to 8.640 ppm. And 95% confidence Interval was 99.14 to 99.97.The detection limit and quantitation limit were established as 0.0096 ppm (0.20%) and 0.048 ppm (1.00%) respectively. At LOQ level the % RSD of % recovery of 6 replicate injections was 4.59% and in accuracy study recovery was 101.52%. The method was found robust for possible changes. The sample solution was found stable up to 48 hours and mobile phase was found stable up to 7 days. Hence this method can be considered to assess the quality of the drug product during stability study and routine analysis with consistent and reproducible results.

1.0 Introduction

Salbutamol sulphate is a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease. It acts by stimulating the adenyl cyclase enzyme, which catalyzes the formation of cyclic-3, 5-adenosine monophosphate (cyclic AMP) from adenosine triphosphate (ATP). The formed cyclic AMP mediates the cellular responses. The increased cyclic AMP levels are associated with relaxation of bronchial smooth muscles. Salbutamol sulphate is effective by oral and inhalation routes of administration.

The Validation concept has been evolving continuously since in first formal appearance in the United States in 1978. Validation is a fast growing and evolving subject. Validation in a requirement that has always made sense from both a regulatory and quality perspective[1,2]. As the analytical process varies so widely there is no universal approach to validation regulatory bodies such as FDA and EC for medicinal products have developed general non-mandatory guidelines[3,4].

The most common reason for validation is to guarantee as far as possible that all processes and machinery in the pharmaceutical manufacturing process are being used in a way which will ensure safety, integrity, quality and strength of the product for use by the general public[5,6]. A literature search[7-11] revealed that very few methods are published for the determination of Salbutamol sulphate and its related substances. However, an isocratic HPLC method[7] and a gradient method[8] are available for the determination of Salbutamol sulphate and its related substances either in raw material, tablets, syrups and/or inhalers. The present HPLC method was validated following ICH guidelines[12].

The present study describes an HPLC method, with a high sensitivity, precision and accuracy for determination of Salbutamol in a metered dose inhaler. The objective of the study is to outline the procedure and evidence whether the stability indicating assay method for the Salbutamol in Salbutamol Pressurized Inhaler by HPLC method is suitable for its intended purpose to establish the quality with a consistent assay results.

2.0 Materials and Methods

2.1 Reagents and Chemicals

HPLC grade Methanol, analytical grade hydrogen peroxide, Hydrochloric acid, Sodium Hydroxide, Ammonium acetate all from Merck, Darmstadt, Germany. Sodium disulfite from Scharlau, Spain. Salbutamol Sulphate EPCRS, Impurity-B EPCRS, Impurity-

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D EPCRS, Impurity-F EPCRS, Impurity-G EPCRS, Impurity-I EPCRS all from EP commission. Salbutamol Sulphate working Standard (WS) was purchased from Cipla, India. Oleic Acid USNF, Dehydrated Alcohol USP, 1,1,1,2 Tetrafluoroethane (HFA 134a) Ph. Grade was respectively from Croda Chemicals, Hayman Ltd, Ineos Fluor Ltd, England. Salbutamol sulphate inhaler samples were collected from a local market of Bangladesh. Purified water was used for the analytical purpose.

2.2 Instruments

A Waters alliance, model-2695, USA equipped with a UV-Visible detector and a triangle for sample preparation was used. The HPLC method uses a Column: Synergi 4µm Polar-RP 80A^0 150mm x 4.6mm with a Pre-column: Synergi 4µm Polar-RP 80A° 4 x 3.0mm. Data was recorded by using Empower software.

2.3 Method Development

2.3.1 Preparation of buffer solution

One gram of ammonium acetate R was dissolved into purified water in a 1 liter volumetric flask to prepare 0.1% w/v buffer solution**.**

2.3.2 Preparation of mobile phase

A mixture of the above buffer solution, and Methanol in the ratio of 75:25 was prepared and the mixture was filtered through 0.45 µ nylon membrane and then degassed.

2.3.3 Chromatographic conditions

Column used in this method: Synergi 4µm Polar-RP 80A^0 150mm x 4.6mm with a Pre-column: Synergi 4µm Polar-RP 80A° 4 x 3.0mm, injection volume was 75µl. Detection was carried out at 225 nm and the flow rate was 1.05 ml/min and the column temperature was 30^{0}C.

2.3.4 Standard solution

12.0 mg of Salbutamol sulphate Working Standard (WS) was taken into 100 ml volumetric flask. 60 ml of diluents was added and sonicated. 10 ml of the solution was taken into 250 ml volumetric flask. Diluent was added to make to volume. The solution contains 4.8 µg/ml Salbutamol sulphate.

2.3.5 System Suitability solution

The standard solution was used as a system suitability solution.

2.3.6 Sample preparation

This test must only be carried out on can which has been filled for at least 14 days. Immediately before the assay, valve was primed by discharging two doses to waste into air. Aerosol canister was washed with methanol and dried for 2 minutes using compressed air.
A stainless steel base plate that has three legs and a central circular indentation with a hole about 1.5 mm in diameter was placed in a small vessel suitable for shaking and add the volume of solvent was added.
The pressurized container was shaken for about 30 seconds and placed in the vessel. 10 deliveries was discharged below the surface of the solvent actuating the valve at intervals of not less than 5 seconds, maintaining the pressurized container in the vertical plane and discharging the pressurized inhalation through the hole in the centre of the base plate. Remove the pressurized container was removed, washed with the diluents and the combined solution and washings was diluted to 50 ml. Further 10 ml of this solution was diluted to 50 ml with the same diluents. Resultant solution was the sample solution. Content of active ingredient was determined by repeating the procedure on the middle 10 and on the last 10 successive combined actuations of the canisters.

2.4 Method Validation

2.4.1 System suitability

The acceptance criteria for system suitability study are - relative standard deviation (%RSD) of the peak area responses for Salbutamol from six standard solution injections should not be not more than 2.0%. The tailing factor and theoretical plate counts in standard solution should not be more than 2.0 and less than 2000 respectively.

2.4.2 Syringe Filter Evaluation study

Study was done by analyzing assay preparation of sample filtered through different syringe filter.

2.4.3 Specificity

For specificity study identification by IR, placebo interference and impurity interference were observed.

2.4.4 Forced degradation study

This study was carried out by solid state exposure (ambient temperature and moisture) and liquid state exposure (acid, base and neutral hydrolysis, oxidation, reduction).

2.4.5 Linearity

The linearity was carried out by observing the correlation coefficient (R^2) value of only Salbutamol without placebo, Salbutamol constant (Placebo variable and, Salbutamol variable (Placebo constant).

2.4.6 Method Precision

To demonstrate method precision, six replicate assay of sample against same standard at 100% of test concentration was carried out and the precision of method was calculated by computing %RSD of six measurements.

2.4.7 Intermediate Precision (Ruggedness)

Test sample of Salbutamol sulphate representing single batch was analyzed by two different analysts on two different equipments, and on two different days. The ruggedness of the test method was calculated by measuring % RSD of six assay results and % RSD of results of two analysts.

2.4.8 Reproducibility

For reproducibility six replicate assay of sample against same standard at 100% of test concentration at different laboratories was carried out and % RSD of six assay results was observed.

2.4.9 Accuracy

Study was carried out by spiking of placebo with Salbutamol over a range of 80%, 100% and 120% (3 concentration/3 replicates each of the total analytical procedure) of test concentration and by measuring the % recovery of each concentration with %RSD of % recovery of each concentration.

2.4.10 Range

Data generated in linearity, precision and accuracy were considered for establishing the range of the analytical method.

2.4.11 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Limit of detection and limit of quantitation was based on signal to noise ratio.

2.4.12 Robustness

Robustness of the method was investigated by varying the concentration of salt in buffer (±10%) (0.09 %, 0.10 %, 0.11 %), ratio of components of mobile phase(Buffer:MeOH=75:25,80:20and

85:85), changing flow rate (± 0.2) (1.05ml/min, 1.25ml/min, 1.45ml/min), wavelength (±3nm)(222mn, 225nm, 228nm), different column(Different brand or lot), column temperature (± 10)(30ºC, 40ºC, 50ºC), unfiltered (Centrifuge at 5000rpm) and filtered test solution.

2.4.13 Stability Study

Bench top and refrigerator solution stability study was carried out up to 48 hours and bench top stability of mobile phase was carried out up to 7 days.

3.0 Results and Discussion

3.1 System suitability

In optimized chromatographic conditions Relative Standard Deviation (%RSD) of area of Salbutamol sulphate, average tailing factor and theoretical plate count were 0.10 (NMT 2.0%), 1.40 (NMT 2.0) 3109 (NLT 2000) respectively. (Table-1)

Table 1: System suitability Study

Determination	Retention Time	Area	Theoretical plate	Tailing factor
1	3.95	451058	3141	1.39
2	3.96	449664	3084	1.38
3	3.95	451126	3123	1.40
4	3.95	449855	3093	1.39
5	3.95	450906	3098	1.40
6	3.95	450189	3098	1.41
Mean(n=6)	3.95	450466	**3109**	**1.40**
Standard Deviation (SD)	0.00	643.6	21.4	0.00
% Relative Standard Deviation (%RSD)	0.10	**0.10**	0.70	0.80

3.2 Syringe Filter Evaluation study

From the study it was observed that the % assay results of 0.20 μm and 0.45 μm syringe filter was closed to each other which indicated that both are suitable for intended use but considering the safety of HPLC we had selected 0.20 μm syringe filter for the assay of Salbutamol in Salbutamol Pressurized Inhaler by HPLC method (Table-2) .

Table 2: Syringe filter Evaluation

Type	Salbutamol			
	Weight Taken (mg)	Area	% Assay	% Assay (Mean)
Standard	12.26	450489		
Placebo Added	10 Actuations	-	-	-
0.20 μm syringe filter	12.26	453783	100.73	100.74 %
	12.26	453864	100.75	
0.45 μm syringe filter	12.26	453810	100.74	100.67 %

3.3 Specificity

Specificity of an analytical method is its ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical Procedures.[13] From the specificity study, it was observed that no peak from placebo and impurities was coeluted at the retention time of Salbutamol and the assay result of spiked sample (with placebo and impurities) was unaffected by the presence of placebo and known impurities (by comparison with the assay results obtained on unspiked sample). The Salbutamol peak passed the peak purity testing (purity angle was lower than the purity threshold) leading to conclusion that the peak was spectrally homogeneous (none of the excipients and impurities coelute with the Salbutamol peak).The method was very specific to determine Salbutamol.

Table-3 (a): Identification (Specificity)-HPLC (Chromatogram)

Sl. No.	Component	Response
1.	Medium(Blank)	No Positive response
2.	Placebo	No Positive response
3.	Salbutamol Sulphate CRS	Positive
4.	Salbutamol Sulphate WS	Positive
5.	Sample (Placebo and Salbutamol)	Positive

Table-3(b): Identification (Specificity)-IR Spectrophotometer (Spectrum)

Sl. No.	Component	Retention Time (Salbutamol)	Response
1.	Placebo	--	No response
2.	Salbutamol Sulphate CRS	4.08	Positive
3.	Salbutamol Sulphate WS	4.07	Positive
4.	Sample (Placebo and Salbutamol)	4.07	Positive
5.	Impurity B EPCRS	5.25	No response
6.	Impurity D EPCRS	11.09	No response
7.	Impurity F EPCRS	36.58	No response
8.	Impurity G EPCRS	1.46	No response
9.	Impurity I EPCRS	42.26	No response

Table 3(c): Interference due to Placebo (Specificity)

S. No	Component	Weight Taken (mg)	RT of Salbutamol (min)	Area	%Assay	Purity Angle	Purity Threshold	Peak Purity (passed / Failed)
1.	Placebo Added	10 Actuation	-	-	-	-	-	-
2.	Standard	12.40	4.07	462898	-	-	-	-
3.	Unspike Sample	12.40	4.08	464810	100.41	0.719	0.972	Passed
4.	Spike Sample-1	12.40	4.07	464938	100.44	0.548	0.712	Passed
5.	Spike Sample-2	12.40	4.08	465182	100.49	0.525	0.601	Passed

Table 3 (d): Interference due to Impurities (Specificity)

S. No	Component	Weight Taken (mg)	RT (min)	Area	% Assay	Purity Angle	Purity Threshold	Peak Purity (Passed / Failed)
1.	Diluent	-	-	-	-	-	-	-
2.	Standard	12.40	4.12	462024	-	-	-	-
3.	Salbutamol (Unspike Sample)	12.40	4.12	459664	99.49%	0.457	0.527	Passed
4.	Salbutamol (Spike Sample)	12.40	4.12	460186	99.60%	0.505	0.595	Passed

3.4 Forced degradation study

From the forced degradation study it was observed and confirmed that no other formulation components and potential degradation product and impurities were coeluting at the retention time of Salbutamol. The Salbutamol peak pass peak purity testing (purity angle was lower than the purity threshold) leading to conclusion that the peak was spectrally homogeneous (none of the excipients and impurities co elute with the Salbutamol peak). (Table-4)

Table 4: Forced degradation study

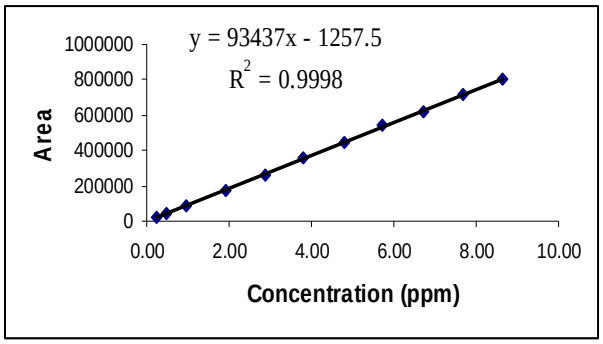
Sr. No	Degradation Condition	Retention Time	Area	% Recovered	Purity Angle	Purity Threshold	Peak Purity (Passed / Failed)
1.	**Solid State (Initial)**	4.04	439792	99.41	0.559	0.614	Passed
1.1	**Exposure to Ambient Temperature and Moisture**						
	After 3 Hours	4.05	457300	101.94	0.395	0.548	Passed
	After 24 Hours	4.06	458617	100.60	0.416	0.532	Passed
1.2	**Exposure to Elevated Temperature (80ºc)**						
	After 3 Hours	4.04	448002	100.35	0.544	0.642	Passed
	After 24 Hours	4.06	438834	97.58	0.382	0.505	Passed
2.	**Liquid State**						
2.1	**Water hydrolysis**						
	Initial	4.12	457838	99.82	0.409	0.501	Passed
	After 2 Hours	4.13	447163	97.49	0.399	0.485	Passed
2.2	**Acid hydrolysis**						
	Initial	4.16	453961	99.44	0.457	0.560	Passed
	After 2 Hours	3.94	439761	96.33	0.353	0.499	Passed
2.3	**Base hydrolysis**						
	Initial	4.60	174361	77.26	0.875	1.073	Passed
	After 2 Hours	4.61	170965	75.76	0.704	0.774	Passed
2.4	**Oxidation**						
	Initial	4.26	195791	86.76	0.831	0.927	passed
	After 2 Hours	4.28	1986	00.88	46.669	90.00	passed
2.5	**Reduction**						
	Initial	4.09	212142	94.00	0.937	1.028	Passed
	After 2 Hours	4.09	670772	29.72	1.666	1.705	Passed

3.5 Linearity

The linearity of an analytical method is its ability to elicit test results directly proportional to the concentration of the analyte in samples within given range[14]. Linearity of peak area response versus concentration was studied on Salbutamol without Placebo, Salbutamol variable (Placebo constant), Salbutamol constant (Placebo variable).The correlation co-efficient obtained was NLT-0.999. (Table-5a, 5b, 5c) (Figure-1, 2 and 3).

Table 5 (a) : Linearity of Salbutamol without Placebo

% Concentration (Salbutamol)	Conc. of Salbutamol Sulphate (ppm) (x-axis)	Mean Area (y-axis)	% Concentration (Salbutamol)	Conc. of Salbutamol Sulphate (ppm) (x-axis)	Mean Area (y-axis)
5%	0.24	21619	100%	4.80	444678
10%	0.48	43100	120%	5.70	539466
20%	0.96	88726	140%	6.72	621006
40%	1.90	174583	160%	7.68	716132
60%	2.88	266218	180%	8.64	806126
80%	3.80	357049			

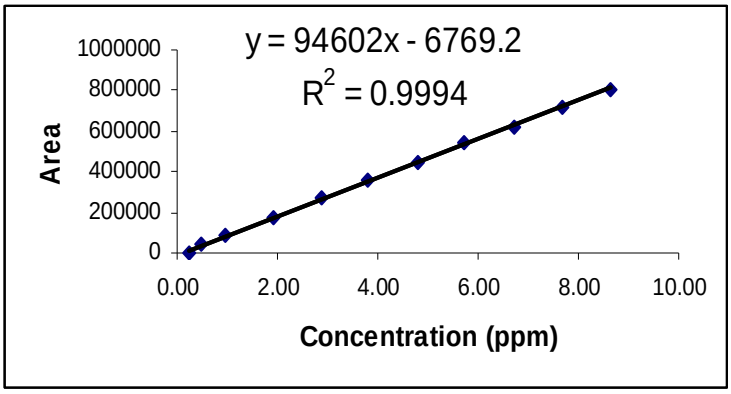


Squared correlation coefficient, R²	0.9998
Slope	93437
Y-intercept	-1257.5
% Y-intercept at the response of 100% level	-0.283%

Figure-1: Graphical Representation of Linearity of Salbutamol without Placebo

Table 5(b): Linearity when Salbutamol variable (Placebo constant)

% Concentration (Salbutamol)	Conc. of Salbutamol Sulphate (ppm) (x-axis)	Mean Area (y-axis)		% Concentration (Salbutamol)	Conc. of Salbutamol Sulphate (ppm) (x-axis)	Mean Area (y-axis)
5%	0.24	22641		100%	4.80	447370
10%	0.48	41485		120%	5.70	539289
20%	0.96	88492		140%	6.72	622927
40%	1.90	176689		160%	7.68	718768
60%	2.88	267632		180%	8.64	807014
80%	3.80	359434				



Squared correlation coefficient, R²	0.9994
Slope	94602
Intercept	6769.2
% Y-intercept at the response of 100% level	-1.51%

Figure 2: Graphical Representation of Linearity when Salbutamol variable (Placebo Constant)

Table 5(c): Linearity when Salbutamol constant (Placebo variable)

% Concentration (Placebo)	Conc. Of Salbutamol Sulphate ppm (x-axis)	Mean Area (y-axis)		% Concentration (Placebo)	Conc. Of Salbutamol Sulphate ppm (x-axis)	Mean Area (y-axis)
20%	4.8	438708		**120%**	4.8	437997
40%	4.8	439254		**140%**	4.8	438551
60%	4.8	438160		**160%**	4.8	438272
80%	4.8	437261		**180%**	4.8	439551
100%	4.8	437869				

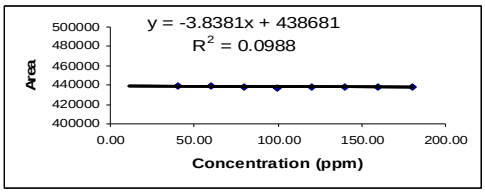


Figure 3: Graphical Representation of Linearity when Sulbutamol constant (Placebo variable)

3.6 Range

The minimum specified range should be considered for the assay of finished product normally from 80 to 120 percent of the test concentration. Based on the linearity, precision and accuracy results, the range of the method was determined as 80% to 120% of the target assay concentration. (Table-6)

Table 6: Range study

Parameter	Salbutamol		
	Concentration Range	Acceptance limit	Result
Linearity	5 % to 180%	R^2 NLT 0.999	R^2 = 0.9998
Precision	100%	% RSD = NMT 2.0	% RSD = 0.679
Accuracy	80% to 120%	%Recovered= 98 % to102%	%Recovered= 99.576%

3.7 Method Precision

The result revealed that % RSD of assay repeatability was 0.6469% and 95% confidence interval was 100.18 to 101.54 which is well within the acceptance limit of 2.0 %. (Table-7a)

Table 7(a): Repeatability study

S. No.	Amount of Sample (mg)	Area	% Assay
1	12.80	444517	101.47
2	12.90	445710	100.96
3	12.90	445356	100.88
4	12.70	441390	101.55
5	11.80	402992	99.79
6	12.90	443763	100.52
Mean			100.86
SD			0.6525
%RSD			0.6469
Standard weight and Area: 11.8 mg and 403834			
95% Confidence Interval : 100.18 – 101.54			

3.8 Intermediate precision or Ruggedness

The results revealed that the % RSD of the assays of two analysts was within the acceptance limit (not more than 2.0) and the individual assay was within 98% to 102% so the stability indicating assay method of Salbutamol considered rugged enough.(Table-7b)

Table 7(b): Table for Intermediate precision or Ruggedness study

Analyst Name	Analyst-1			Analyst-2		
Location	Lab-1			Lab-1		
Instrument used	Waters, Alliance, USA system-1			Waters, Alliance, USA system-2		
Date of analysis	17.04.2010			24.06.2010		
Standard weight and Area: 11.8 mg and 403834				**12.0 mg and 453840**		
Sl. No.	Weight of sample	Area	Assay %	Weight of sample	Area	Assay %
1.	12.80	444517	101.47	12.01	451275	99.35
2.	12.90	445710	100.96	12.15	452315	98.43
3.	12.90	445356	100.88	12.02	452412	99.52
4.	12.70	441390	101.55	12.04	451632	99.18
5.	11.80	402992	99.79	11.99	451138	99.49
6.	12.90	443763	100.52	11.97	451174	99.66
Mean Assay, n=6			100.86	Mean Assay		99.27
Standard deviation, n=6			0.6525	Standard deviation		0.4420
Relative standard deviation, n=6			0.6469%	Relative standard deviation		0.445%
Combined Results of both analysts: Mean assay : 100.0667 Standard Deviation : 0.9856 Relative Standard Deviation : 0.9850%						

3.9 Reproducibility

The reproducibility of the method was evaluated using different analyst and different instrument in the different laboratory. The results reveled that the % RSD of the assays of two analysts was within the acceptance limit (not more than 2.0) so the stability indicating assay method of Salbutamol considered reproducible. (Table-7c)

Table 7(c): Table for reproducibility

Analyst Name		Analyst-1		Analyst-2		
Location		Lab-1		Lab-2		
Instrument used		Shimadzu Class VP, Japan		Waters, Alliance, USA		
Date of analysis		16.04.2010		17.04.2010		
Standard weight and Area: 12.04 mg and 379545				**11.8 mg and 403834**		
Sl. No.	Weight of sample	Area	Assay %	Weight of sample	Area	Assay %
1.	12.02	381575	100.70	12.80	444517	101.47
2.	12.03	381559	100.61	12.90	445710	100.96
3.	12.03	378568	99.83	12.90	445356	100.88
4.	12.01	377651	99.75	12.70	441390	101.55
5.	12.02	380312	100.37	11.80	402992	99.79
6.	12.00	378589	100.08	12.90	443763	100.52
Mean Assay, n=6			100.22	Mean Assay		100.86
Standard deviation, n=6			0.4006	Standard deviation		0.6525
Relative standard deviation, n=6			0.3997%	Relative standard deviation		0.6469%
Combined Results of both analysts: Mean assay : 100.54 Standard Deviation : 0.6134 Relative Standard Deviation : 0.6101%						

3.10 Accuracy

The results of accuracy in terms of % recovery was found to be 99.567%, which was within the acceptance limit of 98% to 102%.(Table-8)

Table 8: Accuracy study

Concentration	**Number of Actuation (placebo)**	**Amount (as Salbutamol) Added X (mg)**	**Peak Area**	**Amount Recovered Y (mg)**	**% RSD**	**% Recovered**	**X^2**	**XY**
80%	12	7.845	341722	7.849	0.26	100.05	61.544	61.574
	12	8.010	343496	7.889		98.49	64.163	63.196
	12	7.928	342906	7.876		99.35	62.847	62.437
Mean		7.928		7.871		99.30		
100%	10	9.909	430146	9.880	0.23	99.70	98.198	97.903
	10	9.992	431898	9.920		99.28	99.841	99.121
	10	9.909	431831	9.918		100.09	98.198	98.286
Mean		9.937		9.906		99.69		
120%	8	11.974	519208	11.925	0.23	99.59	143.38	142.79
	8	11.891	518770	11.915		100.20	141.41	141.69
	8	12.057	520979	11.966		99.25	145.36	144.27
Mean		11.974		11.935		99.68		
		$\sum X$ = 89.516		**$\sum Y$ = 89.139**		**%RSD = 0.54**	**$\sum X^2$ =914.93**	**$\sum XY$ = 911.25**
Standard weight and Area: 12.00 mg and 431443						**95% Confidence interval : 99.14 - 99.97**		
% Recovered = $\frac{(\sum XY) - (\sum X)(\sum Y)}{(\sum X^2) - (\sum X)^2}$ x 100 (where N=9) = 99.576 %								

3.11 Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD based on Signal –to- Noise ratio and it was observed that the signal to noise ratio was 3.5 at 0.0096 ppm. So the detection limit was established as 0.0096 ppm (0.20%).For LOQ the signal to noise ratio is 16.4 at 0.048 ppm. So the quantitation limit was established as 0.048 ppm (1.00%). (Table-9a, 9b, 9c, 9d) and (Figure-4)

Table 9 (a): Table for Limit of Detection

Limit of Detection	**Results (As Salbutamol)**	
	ppm	**%**
Based on Signal –to- Noise ratio	0.0096	0.20

Table 9(b): Table for Limit of quantitation

Limit of Quantitation	**Results (As Salbutamol)**	
	ppm	**%**
Based on Signal –to- Noise ratio	0.048	1.00

Table 9(c): Table for Injection precision at LOQ level

Sl. No.	Amount Added (as Salbutamol) mg	Area	Amount of Sample Recovered	% Assay
Sample 1	9.909	4615	10.206	102.99
Sample-2	9.909	4786	10.584	106.81
Sample-3	9.909	4372	9.668	97.57
Sample-4	9.909	4771	10.551	106.47
Sample-5	9.909	4958	10.964	110.64
Sample-6	9.909	4927	10.896	109.95
Mean		105.74		
SD		4.8545		
%RSD		4.5910		
Standard weight: 12.00 mg and Area: 4481				

Conc. (ppm)	Area
0.048	4481
0.12	11093
0.24	21868

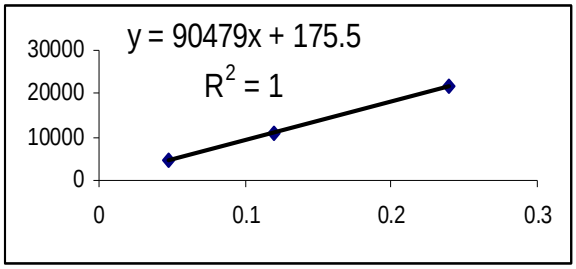


Figure 4: Linearity at LOQ Level

Table 9(d): Table for accuracy at LOQ level

Sl. No.	Amount Added (as Salbutamol) mg	Area	Amount of Sample Recovered	% Recovered
Sample -1	9.909	4665	10.316	104.11
Sample-2	9.909	4318	9.549	96.36
Sample-3	9.909	4664	10.314	104.08
Mean				101.52
SD				4.46
%RSD				4.40
Standard weight: 12.00 mg and Area: 4481				

The above results showed that % RSD of 6 replicate injections was 4.59% which was within the acceptance limit of 10.0% and in accuracy study % recovery was 101.52% at LOQ level.

3.12 Robustness:

Table-10a: Effect of molar concentration of salt in buffer (± 10%)

% w/v Ammonium acetate solution	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
0.09 %	404192	12.10	405970	100.44	4.10	2666	1.45	0.5
0.10 %	403713	12.10	404296	100.14	4.61	2870	1.46	0.4
0.11 %	406983	12.10	404134	99.30	4.03	2686	1.44	0.3

Table 10b: Effect of ratio of components of mobile phase (± 5)

Buffer : Methanol (v/v)	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
75:25	410491	12.10	409570	99.78	3.77	2570	1.46	0.2
80:20	410128	12.10	403917	98.49	4.61	2841	1.43	0.7
85:15	398580	12.10	398354	99.94	5.40	2965	1.41	0.2

Table 10c: Effect of changing flow rate (± 0.2)

Flow rate (ml/min)	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
1.05	481406	12.10	482984	100.33	5.44	3016	1.50	0.2
1.25	403713	12.10	404907	100.30	4.61	2870	1.46	0.4
1.45	348335	12.10	347568	99.78	4.00	2737	1.42	0.2

Table 10d: Effect of different wavelength (± 3 nm)

Wavelength (nm)	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
222	407650	12.10	406871	99.81	4.62	2872	1.45	0.3
225	403793	12.10	402044	99.57	4.61	2882	1.45	0.3
228	348580	12.10	349899	100.38	4.61	2879	1.44	0.3

Table 10e: Effect of different column (brand/lot)

Column temperature (ºC)	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
30 ºC	400130	12.10	402612	100.62	5.09	2725	1.49	0.5
40 ºC	404964	12.10	405117	100.04	4.61	2878	1.46	0.2
50 ºC	406583	12.10	405924	99.84	4.16	2938	1.43	0.2

Table 10f: Effect of column oven temperature (± 10)

Column (brand/lot)	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
Column-1	405283	12.10	404672	99.85	4.58	2870	1.46	0.5
Column-2	380395	12.10	374911	98.56	4.18	2794	1.40	0.3

Table 10g: Effect of unfiltered (Centrifuge) and filtered test solution

Condition of Test Solution	Standard Area	Initial Amount of Sample (mg)	Sample Area	Amount Recovered (%)	Retention Time (min)	Theoretical plate	Tailing Factor	% RSD of Area
Filtered	403713	12.10	405200	100.13	4.61	2870	1.46	0.4
Unfiltered (Centrifuge)	403713	12.10	404253	100.37	4.61	2870	1.46	0.4

The above results demonstrated that there was no significant change in the system suitability parameters and %assay results by changing concentration of salt in buffer (± 10%), changing ratio of buffer and methanol in mobile phase (± 5), changing flow rate (± 0.2), changing wavelength (± 3 nm), changing HPLC column (different lot), changing HPLC column oven temperature (± 10), using both unfiltered (Centrifuge) and filtered test solution, so the method was robust.

3.13 Stability study

From the solution stability study it was observed that the test sample solution was found stable for 48 hours and the mobile phase was found stable for 7 days. (Table-11a, 11b)

Table 11a: Solution stability study (Bench top and refrigerator stability of sample solution)

Time in Hour	Area		% Assay	
	RT	2ºC- 8º C	RT	2ºC- 8ºC
Initial	430033		99.82	
3 Hrs	434767	432588	100.92	100.42
6 Hrs	428665	431959	99.51	100.27
12 Hrs	433185	428901	100.55	99.56
22 Hrs	430147	432546	99.85	100.41
24 Hrs	430331	433785	99.89	100.69
30 Hrs	429584	429321	99.72	99.66
36 Hrs	430916	430207	100.21	100.04
46 Hrs	434419	432123	99.92	99.89
48 Hrs	433269	431746	101.07	100.00
Mean			**100.15**	**100.08**
SD			0.5307	0.3636
% RSD			0.5299	0.3633
Standard weight: 12.10 mg and Area: 430796				

RT: Room Temperature (Bench Top), 2**º C- 8º C:** Refrigerator Temperature

Table 11b: Table for stability study of Mobile Phase

Frequency (Day)	System suitability				Appearance of MP
	Retention Time	%RSD of Area	Tailing Factor	Theoretical plate	
Day-1	4.46	0.20	1.41	2813	Not show any haziness and turbidity
Day-2	4.42	0.30	1.42	2774	
Day-3	4.44	0.50	1.43	2772	
Day-4	4.44	0.10	1.42	2759	
Day-5	4.44	0.10	1.42	2779	
Day-6	4.49	0.40	1.44	2843	
Day-7	4.43	0.30	1.44	2777	

4.0 Conclusion

The stability indicating assay method adopted for the Salbutamol in Salbutamol Inhaler by HPLC method was precise, linear, accurate, rugged and robust enough. The sample solution was found stable up to 48 hours and mobile phase was found stable up to 7 days. Hence this method can be considered for it's intended purpose to establish the quality of the drug product during stability study and routine analysis with consistent and reproducible results.

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