A Novel Validated RP-HPLC Method For the Estimation of Salmeterol By DOE

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ABSTRACT

Background: Salmeterol is a long-acting beta agonist (LABAs) Salmeterol is hypothesized to bind to 2 sites on the beta-2 adrenoceptor. The saligenin moiety binds to the active site of the beta-2 adrenoceptor. The hydrophilic tail of salmeterol binds to leucine residues in the exo-site of the beta-2 adrenoceptor almost irreversibly, allowing salmeterol to persist in the active site, which is responsible for its long duration of action.

Method: A new, simple, accurate, rapid, precise, reproducible and cost-effective RP-HPLC method for the quantitative estimation of Salmeterol in bulk and pharmaceutical dosage form. The developed RP-HPLC method for the quantitative estimation of Salmeterol is based on measurement of absorption at maximum wavelength 254 nm using Phosphate buffer: Methanol P^H 2.5 (35:65 v/v) as a solvent. The stock solution of Salmeterol was prepared, and subsequent suitable dilution was prepared in mobile phase to obtained standard curve. The standard solution shows absorption maxima at 254 nm.

Results: The Salmeterol obeyed Beer Lambert's law in the concentration range of 20-100µg/ml with regression 0.999 at 254 nm. The overall % recovery was found to be 99.78% for Salmeterol which reflects that the method was free from the interference of the impurities and other excipients, used in the bulk and marketed dosage form. The low value of % RSD was indicative of accuracy and reproducibility of the method. The % RSD for inter-day and intra-day precision was found to be 0.2 for Salmeterol respectively which is <2% hence proved that method is precise.

Conclusion: In the present study, novel reverse phase High performance liquid chromatography method for simultaneous determination of Salmeterol in pharmaceutical dosage form was developed. The developed method was validated for various parameters such as accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity as per ICH guidelines.

Keywords: Salmeterol, RP-HPLC, Method development, Validation.

INTRODUCTION

The field of pharmaceutical research continually seeks innovative methods to improve drug development, formulation, and analysis. Salmeterol, a novel and potent bronchodilator, has shown great promise in the treatment of asthma and chronic obstructive pulmonary disease COPD. Accurate and reliable estimation of this drug's concentration is crucial to ensure its efficacy and safety. High-Performance Liquid Chromatography (HPLC) is a widely employed analytical technique for drug quantification due to its high sensitivity, specificity, and reproducibility.

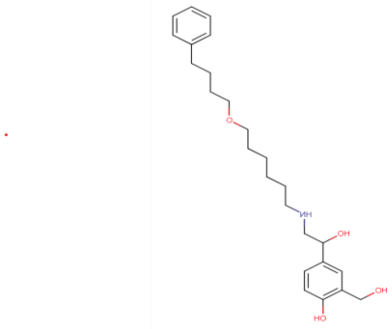

Fig No:1 Structure of Salmeterol

This research presents a novel approach to estimate Salmeterol using a Design of Experiment (DOE). RP-HPLC method in conjunction with hydrotropic solubilization. Design of Experiment is a statistical methodology that allows researchers to optimize experimental parameters systematically, leading to more robust and efficient analytical methods.

The objectives of this study are as follows:

1. To develop an RP-HPLC method for the estimation of salmeterol with improved sensitivity and accuracy by utilizing hydrotropic agents.
2. To employ the Design of Experiment approach to systematically evaluate and optimize critical factors affecting the HPLC analysis, such as mobile phase composition, column temperature, flow rate, and injection volume.
3. To validate the optimized method in accordance with regulatory guidelines, ensuring its reliability and reproducibility for routine analysis.

The significance of this research lies in its potential to provide a more sensitive and efficient HPLC method for the estimation of salmeterol, thus contributing to the quality control of this important antiretroviral drug. Additionally, the utilization of Design of Experiment principles ensures a systematic and thorough exploration of the experimental space, leading to robust and transferable analytical methods.

The remainder of this paper will develop into the methodology, results, and discussion of the experimental findings. Furthermore, the validation and application of the optimized RP-HPLC method for the estimation of salmeterol in real pharmaceutical samples will be presented, along with a comparison to existing methods. Ultimately, this research aims to offer a valuable contribution to the field of analytical chemistry and pharmaceutical analysis.

Experimental:

Chemicals and reagents:

Salmeterol (**Manufacturer** – KP LABS.), Water, Methanol, Acetonitrile, Ortho phosphoric acid and potassium di hydrogen ortho phosphate.

METHOD DEVELOPMENT BY RP- HPLC[8-10]:

HPLC system (Shimadzu) with PDA detector was used. The software LC-Solution can be used and a Rheodyne injection with a 20µL loop was used for injection of the sample. Trerosil C_{18} (100 mm x 4.6 mm)5µg was used. The mobile phase was composed of phosphate buffer: Methanol in the ratio (35:65V/V) with flow rate of 0.8ml/min. HPLC system was operated at ambient temperature.

1. Preparation of Standard Stock Solution

10mg of Salmeterol standard was transferred into 10 ml volumetric flask and 7ml of diluent was added slowly and made up to the mark with diluent to obtain a concentration of 1000µg/ml.

2. Preparation of Working stock solution:

0.6ml of the standard stock solution was pipetted out and transferred into 10ml of volumetric flask and diluted up to the mark with diluent to obtain a concentration of 60µg/ml.

3. Preparation of Mobile phase:

Mobile phase consists of phosphate buffer: Methanol in the ratio (35:65V/V) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter.

4. Design of Experiment:

The standard drug sample of Salmeterol was subjected to the design of experiment process. Box-Behnken response surface design was employed to identify the underlying facts of effects of factors and their interaction effects on selected method responses. A total of 17 runs were conducted.

Statistical analysis:

- By using ANOVA, the statistical calculations were processed for variables screening and optimization of the method.
- The statistical tools provide the numerical verification of variables and its effect on responses

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Method operable design region:

The different amalgamation and reciprocity of input factors produces the space referred as Design space. The establishment of design space was made by utilizing the contour graphs of Sigma tech software.

Method Verification:

The optimized method conditions were proposed by the software in order to reach the desired method goals. The method was verified to check the predictability of the proposed model.

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	1.07	1.08	-0.0086	0.75	-0.665	-0.636	0.133	-1.101	10
2	1.15	1.15	-0.0035	0.75	-0.27	-0.251	0.022	-0.435	7
3	1.14	1.14	0.0023	0.75	0.173	0.161	0.009	0.279	5
4	1.27	1.28	-0.0051	0.75	-0.395	-0.37	0.047	-0.641	1
5	1.27	1.21	0.0592	0.2	2.55	8.874(1)	0.163	4.437(2)	17
6	1.28	1.29	-0.0064	0.75	-0.491	-0.463	0.072	-0.802	2
7	1.33	1.33	0.0029	0.75	0.222	0.206	0.015	0.357	9
8	1.16	1.16	-0.0022	0.75	-0.173	-0.161	0.009	-0.279	8
9	1.2	1.21	-0.0148	0.2	-0.638	-0.608	0.01	-0.304	16
10	1.2	1.21	-0.0148	0.2	-0.638	-0.608	0.01	-0.304	14
11	1.2	1.21	-0.0148	0.2	-0.638	-0.608	0.01	-0.304	15
12	1.03	1.02	0.0064	0.75	0.491	0.463	0.072	0.802	3
13	1.17	1.16	0.0035	0.75	0.27	0.251	0.022	0.435	6
14	1.2	1.21	-0.0148	0.2	-0.638	-0.608	0.01	-0.304	13
15	1.08	1.08	-0.0029	0.75	-0.222	-0.206	0.015	-0.357	12
16	1.05	1.04	0.0051	0.75	0.395	0.37	0.047	0.641	4
17	1.35	1.34	0.0086	0.75	0.665	0.636	0.133	1.101	11

Table: 1 Box - Behnken design experimental runs

Method Validation

1. Linearity:

From the above standard stock solution pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml into a five 10ml volumetric flask and made up to the volume 10ml with diluent to get 2, 4, 6, 8 and 10µg/ml concentrated solutions of was filtered and injected into HPLC system and peak area was measured. Plotted a graph between peak area and concentration. Correlation coefficient was determined by regression analysis.

2. Precision:

From the standard stock solution an aliquot of 0.6ml was added into a six 10ml volumetric flasks, made up to 10ml with diluent. Later it was filtered and six replicates were injected into HPLC system and measured the area for all six injections.

3. Accuracy:

Preparation of standard stock solution:

1000µg/ml of standard stock solution was prepared. Further pipetted out 0.6ml of Standard stock solution into 10ml volumetric flask and was diluted up to the mark with diluent.

Preparation of sample solution:

Accuracy solutions at 50% level:

5mg of sample is weighed and transferred into a 10ml volumetric flask added about 7ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 100% level:

10mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 150% level:

15mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

4. LOD and LOQ:

The limit of detection and limit of quantification was calculated based on the standard deviation of the response and slope of calibration curve.

5. Robustness:

It is the capacity of the method to remain unaffected by small deliberate variations like change in the flow rate and mobile phase composition was made to evaluate the impact on the method.

Flow rate variations:

60ppm of Salmeterol was prepared and injected into HPLC system using the variation in flow rates along with method flow rate, i.e., 0.8ml/min, 1.0ml/min and 1.2ml

Variation in organic composition in mobile phase:

60ppm of salmeterol was prepared and injected into HPLC system using the varied organic composition in mobile phase along with method mobile phase composition i.e., 10% less, Actual and 10% more.

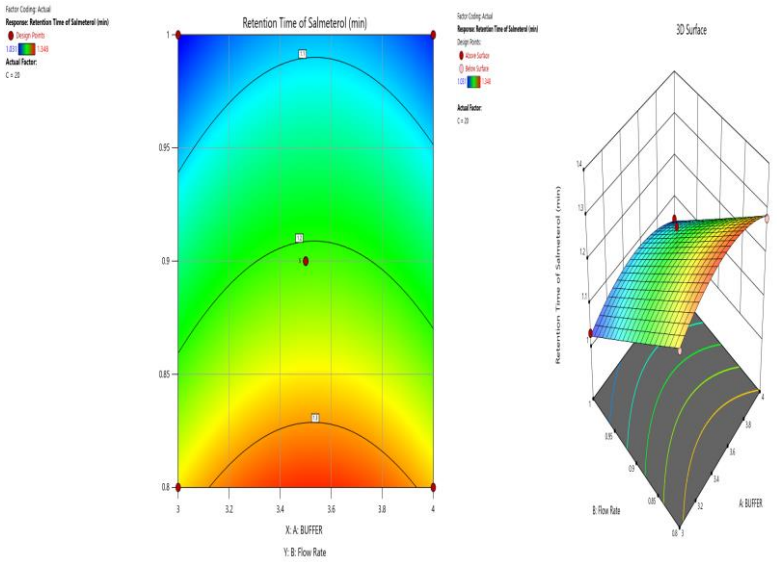
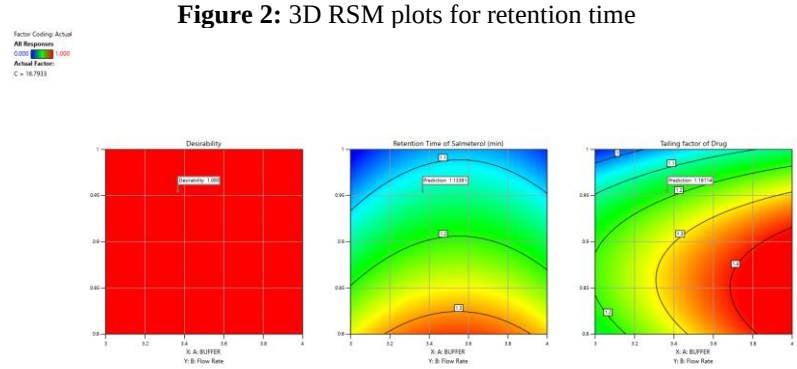


Figure 2: 3D RSM plots for retention time



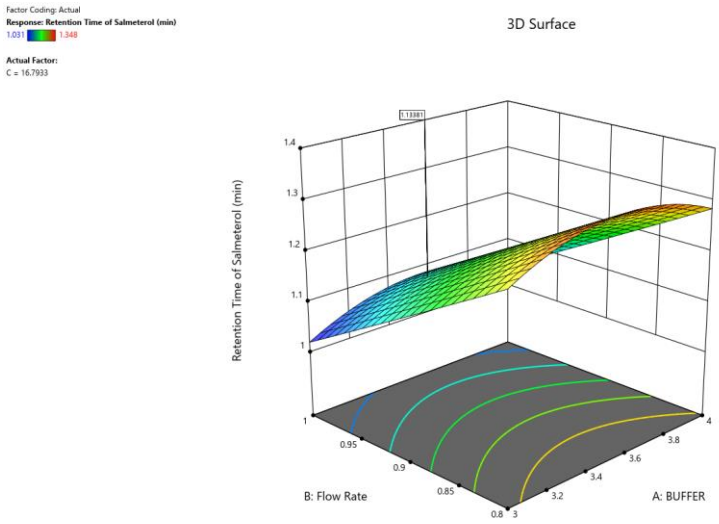


Figure 3: 3D RSM plots for Tailing factor

Optimization and development of RP-HPLC-PDA method using Box-Behnken design

- In the proposed investigation, 17 experimental runs were performed and analyzed for obtained results of retention time and tailing factors in accordance with the Box-Behnken design.
- Further investigation was performed using response surface methodology (RSM) to evaluate the relationship between the dependent responses and independent variables (Factors) using obtained data was reported in Table 1.
- The model was also validated by analysis of variance (ANOVA) using design expert software, and the results are as presented in Table 2. Based on value, a quadratic model was selected for responses such as retention time and tailing factor.
- The significant effects showed p value less than 0.05, while the low standard deviation (% C.V) and a high adjusted R-square value indicated a good relationship between the experimental data and those of the fitted model.
- The predicated R-square value was in acceptance concordance with the adjusted R-square value for all responses.
- The final equation in terms of actual components and factors which can be used to make predictions about the response for given levels of each factor.

Response	Name	Units	Observations	Minimum	Maximum	Mean	Std. Dev.	Ratio
R1	Retention Time of Salmeterol	min	17.00	1.031	1.348	1.18	0.0941	1.31
R2	Tailing factor of Drug		17.00	0.94	1.41	1.21	0.1873	1.50

Table: 2 ANOVA Table

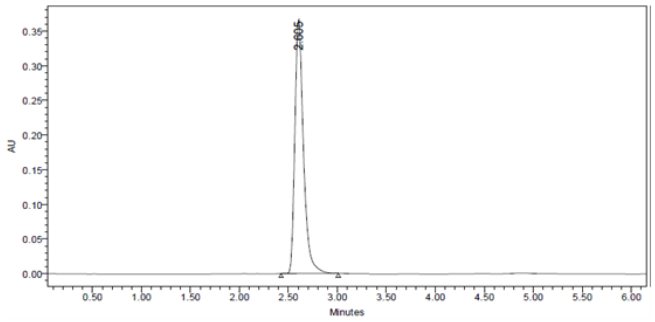


Fig4. : Chromatogram of Optimized method

METHOD VALIDATION:

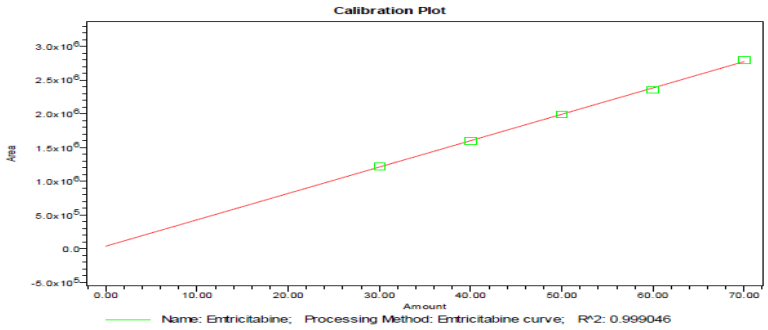


Fig 5: Linearity Curve of salmeterol

S. No	Linearity Level	Concentration	Area
1	I	20	1224140
2	II	40	1595681
3	III	60	**1992966**
4	IV	80	2356546
5	V	100	2797214
Correlation Coefficient			**0.999**

Table :3: Linearity Curve of salmeterol

Acceptance criteria: Correlation coefficient should be not less than 0.999.

ACCURACY:

The accuracy is the method of closeness of the measured value to true value for the sample. Accuracy is usually determined by recovery studies.

Table 4: Accuracy results of Salmeterol

Concentration (At specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1017498.5	5	4.99	99.90	100.58%
100%	1987384.8	10	9.88	98.86	
150%	2992493.4	15	15.08	100.58	

Acceptance criteria:
The % recovery for each level should be between 98.0 to 102.0%.

PRECISION:
The precision studies were carried out by 6 replicate injections of Salmeterol.

Table 5: Precision (Repeatability) results of Salmeterol

Injection	Area
Injection-1	3794064
Injection-2	3800979
Injection-3	3800108
Injection-4	3801140
Injection-5	3814151
Average	**3804150.0**
Standard Deviation	**8287.8**
%RSD	**0.2**

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

Table 6: Intermediate Precision results of Salmeterol

Injection	Analyst 1
Injection-1	2005053
Injection-2	2007362
Injection-3	2007473
Injection-4	2009153
Injection-5	2012800
Average	2008368.1
Standard Deviation	2874.8
%RSD	0.1

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

ROBUSTNESS:
As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

Table 7: Robustness results for Salmeterol (Change in Flow rate)

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	5752	0.99
2	**1.0***	5026.5	1.1
3	1.2	4476	1.02

Table 8: Robustness results for Salmeterol
(Change in Organic Composition in the Mobile Phase)

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	5% less	6498	1.2
2	*Actual	5026.5	1.3

3	5% more	6471	1.2

ASSAY:

$$Assay\ \% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

= 99.87

Where, Avg.wt = average weight of tablets
P= Percentage purity of working standard
LC= Label Claim of Salmeterol mg/ml

Table 9: Assay

ASSAY	1065799	10	0.6	10	10	99	295.75	100	**99.87**
	3793522	10	10	10	0.6	100	25		

Discussion: The percentage assay of Salmeterol was found to be 99.87%.

Table 10: Summary data of validation parameters

S.NO	VALIDATION PARAMETERS		ACCEPTANCE CRITERIA	RESULTS
1.	System suitability		%RSD for 5 replicate injections of standard solution NMT 2.0%	0.1
2.	Linearity		The correlation coefficient should be NLT 0.999	R^2=0.999
3.	Accuracy		The %Recovery at each level should be NLT 80.0% and NMT 120% of the amount added	%Recovery – 100.59
4.	Precision		The %RSD of peaks obtained from the 6 replicate injections should be NMT 2.0%	%RSD- 0.2
5.	LOD		-	0.001µg/ml
6.	LOQ		-	0.004µg/ml
7.	Robustness	Variation in flow rate(0.8ml/min-1.2ml/min)	%RSD should be NMT 2	%RSD 0.8ml/min – 0.00132 1.2ml/min – 0.00587
8.	Assay		-	99.87%

Conclusion

In the present study, novel reverse phase High performance liquid chromatography method for simultaneous determination of Salmoterol in pharmaceutical dosage form was developed. The developed method was validated for various parameters such as accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity as per ICH guidelines.

A) Method development:

- Trial 6 was optimized for the method development of deliberately changing the chromatographic conditions.
- Column used was Trerosil C_{18} (100 mm x 4.6 mm)5µg. mobile phase composition of buffer: Methanol in the ratio (35:65V/V) and buffer pH 2.5 adjusted with ortho phosphoric acid. The Flow rate set to 0.8ml min^{-1} with UV detection was carried out at 254 nm.

B) Validation Parameters

- The calibration was linear with correlation coefficient 0.999 for Salmoterol
- In precision it was found that % RSD is less than 2% which indicates that the Proposed method has good reproducibility.
- The system suitability parameter indicates good resolution of both the peaks > 2.
- From the Accuracy was found that % Recovery of the drug was found to be in the range of % for Salmeterol.
- Robustness, when pH was altered RT has no changed significantly, when mobile phase was altered there was no change in the RT significantly.

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