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DEVELOPMENT AND VALIDATION OF A REVERSED-PHASE HPLC METHOD FOR ANALYSIS OF TIOTROPIUM BROMIDE AND SALMETEROL XINAFOATE IN METERED DOSE INHALATION FORM

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ABSTRACT

A simple, sensitive, rapid and reproducible reversed- phase HPLC method has been developed and validated for estimation of Tiotropium bromide and Salmeterol xinafoate. The assay involved an isocratic elution of these two components on Kromosil C₁₈ column (150 X 4.6 mm, 5µm) using a mobile phase composition of Buffer: Acetonitrile: Methanol (55:35:10). The flow rate was 1.5 mL/min; Column oven temperature 50°C and the analytes monitored at 235nm. Calibration curves were linear with coefficient correlation between 0.99 to 1.0 over a concentration range of 0.447 to 6.705 µg/mL of Tiotropium bromide and 0.072 to 1.081 µg/mL for Salmeterol xinafoate respectively. All the validation parameters were within the acceptance range according to ICH norms. The Method has been successfully applied for analysis of drugs in pharmaceutical formulation. Results of analysis were validated statistically and by recovery studies.

Key words Tiotropium bromide; Salmeterol fumarate; Reversed-phase HPLC; Antiasthma tic.

INTRODUCTION

Tiotropium Bromide is an anticholinergic bronchodilator used in the management of chronic obstructive pulmonary disease (COPD) [1]. It is a muscarinic receptor antagonist, often referred to as an antimuscarinic or anticholinergic agent. Although it does not display selectivity for specific muscarinic receptors, on topical application it acts mainly on M3 muscarinic receptors located in the airways to produce smooth muscle relaxation, thus producing a bronchodilatory effect [2].

Salmeterol xinafoate (SX) is, (RS)-2-(hydroxymethyl)-4-{1-hydroxy-2-[6-(4-phenylbutoxy) hexylamino] ethyl} phenol. It is a long acting and highly selective β₂ agonist formulated as its 1-hydroxy-2-napthoate (xinafoate) salt used in the treatment of asthma and chronic obstructive pulmonary disease. Inhaled salmeterol works like other beta 2-agonists, causing bronchodilation by relaxing the smooth muscle in the airway so as to treat the exacerbation of asthma. The long duration of

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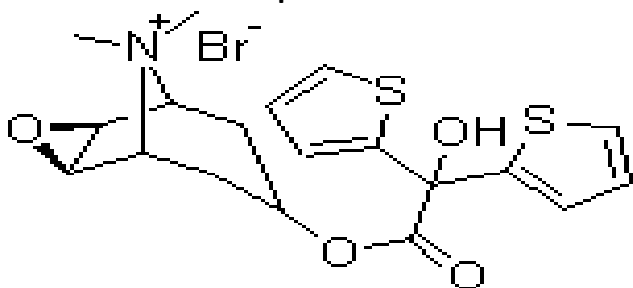
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action occurs by the molecules initially diffusing into the plasma membrane of the lung cells, and then slowly being released back outside the cell where they can come into contact with the beta-2 adrenoceptors, with the long carbon chain forming an anchor in the membrane [3].

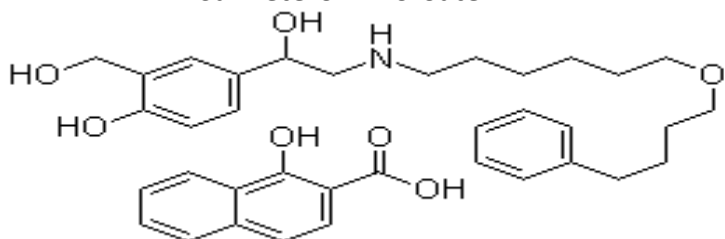
The aim of this study Tiotropium bromide and Salmeterol xinafoate is latest combination of anti asthmatic drugs. It is available in dry powder inhalation and metered dose inhalation form. Both the drugs were individually official in Indian pharmacopoeia [4], United States pharmacopoeia [5] and British pharmacopoeia [6].

Literature survey revealed spectrophotometric and chromatographic methods reported for estimation of Tiotropium bromide and Salmeterol xinafoate individually or in combination with other drugs. However, there is no analytical method reported for the simultaneous determination of these drugs in a pharmaceutical formulation. Present work describes simple, rapid, accurate and precise method for simultaneous determination of Tiotropium bromide and Salmeterol xinafoate in metered dose inhalation. The proposed methods were validated as per ICH guidelines [7].

Tiotropium Bromide



Salmeterol Xinafoate



MATERIALS AND METHODS

Chemicals and reagents:

Working standards of pharmaceutical grade Tiotropium bromide and Salmeterol xinafoate were

obtained as generous gifts from Hetero Labs Ltd (Hyderabad, India) and was used as such without further purification. The pharmaceutical dosage form used in this study was Hetero R&D Inhalation Aerosol (Tiotropium bromide and Salmeterol xinafoate) 120 Metered Dose. Acetonitrile and Methanol (HPLC Grade), Triethylamine (AR Grade), Orthophosphoric acid (AR Grade) purchased from Merck specialties Pvt.Ltd, (Mumbai, India) and double distilled water were used in analysis.

Instrumentation and Chromatographic conditions:

Shimadzu HPLC system LC-2010 CHT consisting of UV/VIS detector and LC solutions software was used for analysis. Separation was carried out on Kromosil C₁₈ (150 x 4.6 mm i.d.) column using Buffer pH 2.5: Acetonitrile: Methanol in ration of (55:35:10 v/v) as mobile phase at flow rate of 1.5 ml/min and Column oven temperature 50°C. Samples were injected using auto injector with 100µL loop and detection was carried out at 235 nm. All weighing were done on Shimadzu balance (Model AY-120).

Preparation of Buffer (pH 3.5)

Pipette 2.0 ml of Triethylamine into a beaker containing 1000 ml of water and dissolve it. Adjust the pH of the solution to 2.5 ± 0.05 with orthophosphoric acid and mix. Filter through 0.45µm membran filter.

Preparation of Diluent

Prepare a mixture of 90 volumes of 0.05% v/v ortho- phosphoric acid and 10 volumes of Acetonitrile.

Preparation of standard stock solutions:

Tiotropium bromide standard stock solution:

Accurately weighed and transferred 22.5 mg of Tiotropium bromide working standard into a 100 ml volumetric flask. Added 50 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transferred 10 ml of the above solution in to a 100 ml volumetric flask and dilute to volume with diluent.

Salmeterol xinafoate standard stock solution:

Accurately weighed and transferred 18.5 mg of Tiotropium bromide working standard into a 100 ml volumetric flask. Added 50 ml of diluent and sonicate to dissolve. Dilute to volume with diluent

and mix. Transferred 10 ml of the above solution in to a 100 ml volumetric flask and dilute to volume with diluent.

Preparation of standard solutions:

Transferred 2.0 ml of Tiotropium bromide standard stock solution and 5.0ml of Salmeterol xinafoate standard stock solution into a 100 ml volumetric flask dilute to volume with diluent.

Preparation of sample solution A:

Took a container, fit in an actuator & prime the valve by wasting first two actuations. Shacked the container for at least five seconds in-between each actuation. Remove the container from its actuator & wash with methanol. Clean the valve and valve stem internally and externally with an airline fitted with a narrow jet. Place a disc in clean and dry 100 ml beaker; add about 50 ml of acetonitrile. Hold the container in inverted position, shake for 5 seconds and deliver one actuation in beaker through the hole provided in the center of the disc. Similarly deliver further nine actuations in the same beaker with constant shaking for at least five seconds in between each delivery.

Transferred the solution from beaker into a 100-ml volumetric flask. Wash the beaker and disc with acetonitrile and transferred into the same volumetric flask. Make up the volume up to the mark with acetonitrile. Transferred 10 ml of the above solution in to 25ml volumetric flask, dilute to volume with diluent and mix.

Sample Preparation for Actuator Retention B:

Washed the actuator with mobile phase and dry gently. Fit the container in actuator & deliver 10 actuations with constant shaking for at least 5 seconds in between each delivery. Remove the container from actuator, wash the actuator 3-4 times with 10 ml acetotrile, collect in 25 ml volumetric flask & then make up the volume up to the mark with diluent.

After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the metered dose sample solution A and actuator retention B solution was injected, chromatogram was obtained and the peak areas were recorded. The injections were repeated six times and the amount of each drug present per Available online on www.ijprd.com

inhalation was estimated from the respective calibration curves.

Content of Active Ingredient Delivered per actuation = μg of Sample A - μg of Sample B

% Labeled Amount of Active Ingredient Delivered per actuation

= $[\mu\text{g}$ of Sample A - μg of Sample B] x100

Label Claim

System suitability:

The system suitability was assessed by five replicate injections of the Tiotropium bromide containing 0.450 $\mu\text{g}/\text{ml}$ and Salmeterol xinafoate containing 0.925 $\mu\text{g}/\text{ml}$ of both the drugs. The peak asymmetry, number of theoretical plates, the percentage relative standard deviation of standard solution five injections were calculated as represented in **Table 1**. The values obtained demonstrated the suitability of the system for the analysis of these drugs in combination.

Table 1: System Suitability parameters for RP-HPLC method

Parameters	Values	
	Tiotropium bromide	Salmeterol xinafoate
Theoretical plates	3247	8561
Asymmetry Factor	1.20	1.40
%RSD	0.06	0.85

METHOD VALIDATION

The method was validated for linearity, accuracy, intra-day and inter-day precision and robustness, in accordance with ICH guidelines.

Linearity:

To evaluate linearity of the method, six calibration standards of Tiotropium bromide containing 0.447, 1.118, 2.235, 4.470, 5.588, and 6.705 mg/mL and Salmeterol xinafoate containing 0.072, 0.144, 0.360, 0.721, 0.901, and 1.081 mg/mL were analyzed. A plot of peak areas versus Tiotropium bromide and Salmeterol xinafoate concentrations was linear in the range from 0.447 to 6.705 mg/mL of Tiotropium bromide and 0.072 to 1.081 mg/mL of Salmeterol xinafoate with a correlation

coefficient of 0.9998 and 0.9996. This result demonstrates linearity of this method over the specified range.

Precision:

One set of three different concentrations of mixed standard solutions of Tiotropium bromide and Salmeterol xinafoate were prepared. All the solutions were analyzed thrice, in order to record any intraday variations in the results. For inter day variations study three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days. The peak areas were recorded and relative standard deviation (RSD) was calculated.

Accuracy:

To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100% and 150%. The percentage of recoveries were calculated, results of which are represented in **Table 2**.

Table 2: Recovery studies of Tiotropium bromide (TB) and Salmeterol xinafoate (SX)

Drug	% Level	Amount added($\mu\text{g}/\text{ml}$)	Amount found($\mu\text{g}/\text{ml}$)	% Recovery*	% RSD
TB	50	0.0438	0.0443	100.9	0.29
	100	0.0876	0.0894	101.4	1.31
	150	0.1314	0.1285	97.6	0.41
SX	50	0.1242	0.1239	99.8	0.47
	100	0.2484	0.2472	99.4	0.56
	150	0.3727	0.3811	102.4	0.35

*Average of three determinations.

Limit of Detection and Limit of Quantification:

LOD and LOQ were calculated as $3.3 \sigma/S$ and $10 \sigma/S$ respectively; where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot.

Robustness:

In the robustness study, the influence of small, deliberate variations of the analytical parameters Available online on www.ijprd.com

on retention time of the drugs was examined. The following three factors were selected for change: flow rate of the mobile phase (1.5 ± 0.04 ml/min), a wavelength at which the drugs were recorded (235 ± 1 nm). One factor at the time was changed to estimate the effect. A number of replicate analyses ($n=3$) were conducted at 3 levels of the factor (-, 0, +). It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

RESULTS AND DISCUSSION

For RP-HPLC method different mobile phases were tried and the mobile phase containing Buffer: Acetonitrile: Methanol (55:35:10, v/v) was found to be optimal for obtaining well defined and resolved peaks with mean retention times 1.214 ± 0.01 and 5.101 ± 0.02 min (Mean \pm S.D.) for Tiotropium bromide and Salmeterol xinafoate respectively. The representative chromatogram of the standard solution of mixture is shown in **Figure 1**.

Figure 1: Representative chromatogram obtained for standard mixture of Tiotropium bromide ($0.45 \mu\text{g}/\text{ml}$, 1.214 ± 0.01 min), Salmeterol xinafoate ($0.925 \mu\text{g}/\text{ml}$, 5.101 ± 0.02 min)

Results were found to be linear in the concentration range of 0.447 to $6.705 \mu\text{g}/\text{mL}$ of Tiotropium bromide and 0.072 to $1.081 \mu\text{g}/\text{mL}$ for Salmeterol xinafoate respectively. The correlation coefficients for the plots were 0.9998 for Tiotropium bromide and 0.9996 for Salmeterol xinafoate. The proposed method was also evaluated by the assay of commercially available Inhalations containing Tiotropium bromide and Salmeterol xinafoate. The % assay was found to be 101.3 ± 1.041 for Tiotropium bromide and 96.8 ± 0.012 for Salmeterol xinafoate (mean \pm S.D., $n = 6$). The method was found to be accurate and precise, as indicated by recovery studies and % RSD not more than 2. Robustness of the method (data not shown), checked after deliberate alterations of the analytical parameters shown no marked changes in the chromatogram ($\text{RSD} < 2$), which demonstrated that the RP-HPLC method developed

is robust. The summary of validation parameters of proposed HPLC method is given in **Table 3**.

Table 3: Summary of validation parameters of proposed RP-HPLC method

Parameters	Tiotropium bromide	Salmeterol xinafoate
Linearity range ($\mu\text{g/ml}$)	0.447-6.705	0.072-1.081
Correlation co-efficient	0.9998	0.9996
Slope(m)	18353.68	109752.59
y-Intercept(c)	1082.03	331.79
Limit of detection ($\mu\text{g/ml}$)	0.01	0.100
Limit of quantification ($\mu\text{g/ml}$)	0.02	0.200
Accuracy (% recovery)	97.6-101.4	99.4-102.4
Precision(%RSD)		
Intraday (n =3)	0.301	1.052
Inter day (n =3)	0.528	0.952

CONCLUSION

The validated RP-HPLC method employed here proved to be simple, fast, accurate, precise and robust, thus can be used for routine analysis of Tiotropium bromide and Salmeterol xinafoate in combined metered dosage inhalation form.

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