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Research Article

DEVELOPMENT AND VALIDATION OF NOVEL RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOSALBUTAMOL AND IPRATROPIUM BROMIDE IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A rapid and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of Levosalbutamol and Ipratropium bromide. Chromatographic separation was achieved on reverse phase Enable C_{18} column (250 X 4.6 mm, 5 µm) using the mobile phase consisting of Méthanol: 0.01M potassium dihydrogen phosphate (pH was adjusted to 3.0 with O-phosphoric acid). The mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 245 nm. The proposed method was found to be simple, fast, accurate, precise and reproducible and could be applied for routine quality control analysis for simultaneous determination of Levosalbutamol and Ipratropium bromide in pharmaceutical dosage forms.

Keywords: Levosalbutamol, Ipratropium bromide, RP-HPLC, Validation.

INTRODUCTION

Levosalbutamol[1] (Figure 1) relaxes the smooth muscles of all airways, from the trachea to the terminal bronchioles. Increased cyclic AMP concentrations are also associated with the inhibition of the release of mediators from mast cells in the airways. Levosalbutamol acts as a functional agonist that relaxes the airway irrespective of the spasmogen involved, thereby protecting against all bronchoconstrictor challenges. While it is recognized that beta$_2$-adrenergic receptors are the predominant receptors on brochial smooth muscle, data indicate that there are beta-receptors in the human heart, 10-50% of which are beta adrenergic receptors. Chemically it is 4-[(1R)-2-(tert-butylamino)-1-hydroxyethyl] - 2-(hydroxymethyl) phenol.

Ipratropium Bromide[2] (Figure 2) antagonizes the action of acetylcholine by blocking muscarinic cholinergic receptors resulting in bronchodilation and drying of respiratory tract secretions. Ipratropium blocks muscarinic acetylcholine receptors, without specificity for subtypes, and therefore promotes the degradation of cyclic guanosine monophosphate (cGMP), resulting in a decreased intracellular concentration of cGMP. Most likely due to actions of cGMP on intracellular calcium, this results in decreased contractility of smooth muscle in the lung, inhibiting bronchoconstriction and mucus secreti on. It is a nonselective muscarinic antagonist, and does not diffuse into the blood, which prevents systemic side effects. Ipratropium is a derivative of atropinebut is a quaternary amine and therefore does not cross

the blood brain barrier, which prevents central side effects (anticholinergic syndrome). Chemically it is [8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1] oct-3-yl] 3-hydroxy-2-phenyl-propanoate.

Literature survey reveals that few spectrophotometric and chromatographic methods [3-25] were reported for estimation of Levosalbutamol and Ipratropium bromide in single and combination with other drugs. Therefore an attempt has been made to develop and validate simple, sensitive, precise and accurate RP-HPLC method for simultaneous estimation of Levosalbutamol and Ipratropium bromide in combined respule dosage form. Hence, a combined dosage form of Levosalbutamol and Ipratropium bromide and can be considered as a novel avenue for research.

EXPERIMENTAL

Chromatographic Conditions

The chromatographic separation was achieved on Schimadzu HPLC consisting of isocratic binary pump, PDA detector and thermostat column compartment connected to LC solutions software. The instrument is equipped with a LC 20AD pump and variable wavelength programmable UV-Visible detector, SPD-M20A. A 20µL Rheodyne syringe was used for injecting the samples. Data was analyzed by using LC solutions software. Shimadzu UV-1800 UV-Visible spectrophotometer was used for spectral studies. Data was analyzed by using UV Probe software. Degassing of the mobile phase was done by using a Loba ultrasonic bath sonicator. A Shimadzu balance was used for weighing the materials. The analysis of the drug was carried out on Enable C_{18} column (250 x 4.6 mm; 5 µm), flow rate 1.0 mL/min, wave length 245 nm, column temperature 30°C and run time 8 minutes.

Chemicals and Solvents

The working standards of Levosalbutamol and Ipratropium bromide were provided as gift samples from Dr.ReddyLabs, Hyderabad, India. Levosalbutamol and Ipratropium bromide branded drug Duolin respules were purchased from local market. Potassium dihydrogen phosphate and orthophosphoric acid of AR Grade were obtained from S.D. Fine Chemicals Ltd., Mumbai, India. HPLC grade methanol and acetonitrile was purchased from E. Merck (India) Ltd., Mumbai, India. HPLC grade water obtained from Milli Q water purification system was used throughout the study.

Preparation of mobile phase and diluents

Preparation of Buffer

About 1.36gm of sodium dihydrogen orthophosphate and were transferred into a 1000 ml volumetric flask containing 700 ml of water. The contents were shake for about 5 minutes and the volume made upto 1000 ml with water. This solution was mixed and pH was adjusted to 4.0 with orthophosphoric acid and filtered through 0.45µ nylon filter. The same mobile phase was used as diluent.

Preparation of mobile phase

A mixture of Methanol: 0.01M Di-potassium hydrogen phosphate (pH was adjusted to 3.0 with O-phosphoric acid) in the ratio of 50:50 v/v was prepared and used as the mobile phase.

Preparation of standard stock solution

About 100 mg of Ipratropium bromide and 250 mg of Levosalbutamol were weighed and transferred into a 100 mL volumetric flask containing 25 mL of water. The solution was stirred for 5 minutes and made up with a further quantity of the mobile phase to get 1mg/mL and 2.5mg/mL Ipratropium bromide and Levosalbutamol respectively. This solution was further diluted to get required concentrations during study.

Preparation of sample solution

Commercial respules (DUOLIN) was purchased from the Local market and about 25ml of sample solution was transferred to a 50mL volumetric flask containing 25 mL of the water. The contents of the flask were sonicated for about 20 min for complete solubility of the drug and the volume was made up to 50 mL with mobile phase. Then the mixture was filtered through 0.45µ membrane filter. Above solution was further diluted and 20 µL was then injected six times into the column. The mean peak areas of the drugs were calculated and the drug content in the formulation was calculated

U.V Detection wavelength

The spectra of diluted solutions of the Levosalbutamol and ipratropium bromide were recorded separately on UV spectrophotometer. The peaks of maximum absorbance wavelengths were observed. The spectra of the both Levosalbutamol and ipratropium bromide

were showed that a balanced wavelength was found to be 245 nm.

Method validation

The developed analytical method was validated as per ICH guidelines[7] for the parameters like linearity, accuracy, precision, ruggedness, specificity and system suitability.

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of drug in the samples within a given range. Linearity was performed by preparing mixed standard solutions of Levosalbutamol and Ipratropium bromide at different concentration levels including working concentration mentioned in experimental condition i.e. 5 and 2µg/mL for Levosalbutamol and Ipratropium bromide respectively. Twenty microliters of each concentration was injected in duplicate into the HPLC system. The response was read at 245 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The regressions of the plots were computed by least square regression method From the stock solutions of Levosalbutamol and Ipratropium bromide 0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml, 0.6ml is taken in six different 10 ml volumetric flasks and diluted with the mobile phase to the give the following Concentrations.

Levosalbutamol: 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml,12.5 µg/ml,15 µg/ml.
Ipratropium bromide: 1 µg/mL, 2 µg/ml, 3 µg/ml, 4 µg/ml, 5 µg/ml, 6 µg/ml.

These Solutions were injected six times into the chromatographic system and response was recorded. The calibration graph was plotted with mean peak area on Y axis and concentration of standard solution on X axis. The degree of linearity was estimated by calculating the correlation coefficient. Y- Intercept, slope of the regression line. Beer's law was found to be obeyed over this concentration range. The correlation coefficient shall not be less than 0.998. Linearity results were presented in Table 1 and 2.

Accuracy

Accuracy indicates the deviation between the mean value found and the true value. The accuracy of the method was determined by standard addition method by means of recovery experiments. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and %RSD at each level was calculated and results were presented in Table 3 and 4. Satisfactory recoveries ranging from 99.47 to 100.16 and 99 to 99.6 for Levosalbutamol and Ipratropium bromide respectively were obtained by the proposed method. This indicates that the proposed method was accurate.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions. Precision of the method was performed as system precision, method precision and intermediate precision.

System precision

To study the system precision, six replicate mixed standard solutions of Levosalbutamol and ipratropium bromide were injected. The percent relative standard deviation (% RSD) was calculated and it was found to be 0.71and 0.72 for Levosalbutamol and ipratropium bromide respectively, which are well within the acceptable criteria of not more than 2.0.

Method precision

The method precision study was carried out on six preparations from the same respules samples of Levosalbutamol and ipratropium bromide and percent amount of both were calculated. The %RSD of the assay result of six preparations in method precision study was found to be 0.89 and 0.75 for Levosalbutamol and ipratropium bromide respectively, which are well within the acceptance criteria of not more than 2.0.

Robustness

The robustness study was performed by slight modification in flow rate of the mobile phase, pH

of the buffer and composition of the mobile phase. Mixed samples of Levosalbutamol and Ipratropium bromide at a concentration of 5µg/mL and 2µg/mL respectively were analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature Robustness of method was carried out with variation of Wavelength(243,247), variation in mobile phase composition (±2).

Sensitivity

The limit of detection (LOD) was determined as lowest concentration giving response and limit of quantification (LOQ) was determined as the lowest concentration analyzed with accuracy of the proposed RP-HPLC method. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.31µg/mL and 0.98µg/mL for Levosalbutamol and 0.11µg/mL and 0.37µg/mL for Ipratropium bromide. The LOD and LOQ showed that the method is sensitive for Levosalbutamol and Ipratropium bromide.

System suitability test

The specificity of this method was determined by complete separation of Levosalbutamol and Ipratropium bromide. The tailing factor for peaks of Levosalbutamol and Ipratropium bromide was less than 2% and resolution was satisfactory. The average retention time for Levosalbutamol and Ipratropium bromide were found to be 2.11 and 5.19 respectively, for six replicates. The peaks obtained for Levosalbutamol and Ipratropium bromide were sharp and have clear baseline separation. Analysis was also performed for active Levosalbutamol and Ipratropium bromide, as well as placebo sample at different conditions. After analysis it was found that there is no interference of peak in the Levosalbutamol and Ipratropium bromide region for the placebo & active sample. Hence the developed method was specific for the analysis of this product**.** The system suitability parameters are given in Table 9.

Estimation of Levosalbutamol and Ipratropium bromide in respule dosage forms

Estimation of Levosalbutamol and Ipratropium bromide in respule dosage forms by the developed RP-HPLC method was carried out. The assay procedure was performed and the assay percentage was calculated and presented in the table 6.13.The solution was injected into the column six times. The average peak area of the drug was computed from the chromatograms and the amount of the drug present in the respule dosage form was calculated by using the regression equation obtained for the pure drug.

RESULTS AND DISCUSSION

The present study was aimed at developing a simple, sensitive, precise and accurate HPLC method for the simultaneous analysis of Levosalbutamol and Ipratropium bromide from bulk samples and their Respules dosage forms. A non-polar C_{18} analytical chromatographic column was chosen as the stationary phase for the separation and simultaneous determination of Levosalbutamol and Ipratropium bromide. Mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases. The choice of the optimum composition is based on the chromatographic response factor, a good peak shape with minimum tailing. A mixture of buffer and Methanol in the ratio of 50:50 v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was well defined, better resolved and almost free from tailing. The retention times of the Levosalbutamol and Ipratropium bromide were found to be 2.11 and 5.19 min respectively. The linearity was found satisfactory for Levosalbutamol and Ipratropium bromide in the range 2.5 – 15 µg/mL and 1-6 µg/mL respectively (Table 1 & 2). The regression equation of the linearity curve between concentrations of Levosalbutamol and Ipratropium bromide over its peak areas were found to be Y = 27768X – 8957 (where 'Y' is the peak area and X is the concentration of Levosalbutamol in µg/mL) and Y = 80469X – 15617 (where Y is the peak area and X is the concentration of Ipratropium bromide in µg/mL) respectively. Precision of the method was studied by repeated injection of tablet solution and results showed lower %RSD values (Table 3 & 4). This reveals that the method is quite precise. The percent recoveries of the drug solutions were studied at three different concentration levels. The percent individual recovery and the %RSD at each level were within the acceptable limits (Table 5 & 6). This indicates that the method is accurate. The absence of additional peaks in the chromatogram indicates non-interference of the commonly used excipients in the tablets and hence the method is specific.

The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust (Table 7 & 8). The system suitability studies were carried out to check various parameters such as theoretical plates and tailing factor. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive (Table 9).

CONCLUSION

The proposed method was simple, specific and sensitive and can be used for simultaneous analysis Levosalbutamol and Ipratropium bromide in bulk samples and its respules dosage forms.The result of the study follows the protocol of ICH guidelines and it can be successfully applied for the simultaneous estimation of the marketed products of Levosalbutamol and Ipratropium bromide in bulk samples and its respules dosage forms.

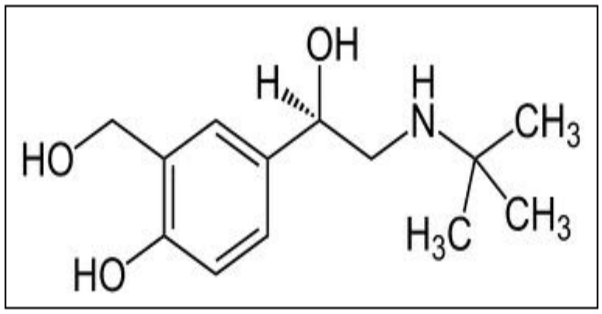

Fig. 1: Chemical structure of Levosalbutamol

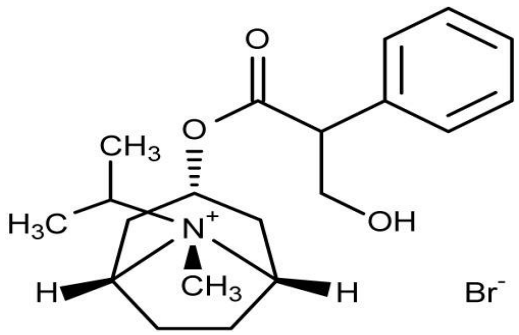

Fig. 2: Chemical structure of Ipratropium bromide

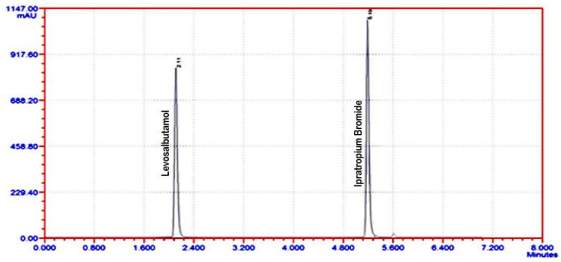

Fig. 3: Typical Chromatogram of standard solution

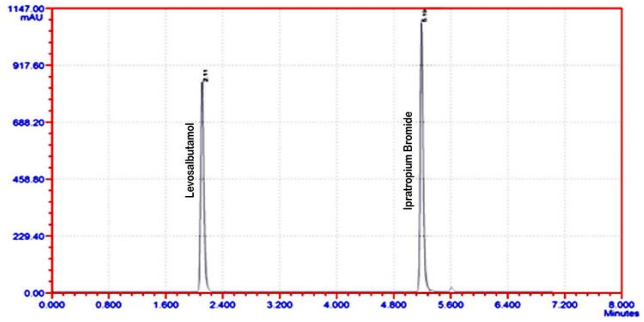

Fig. 4: Typical Chromatogram of sample solution

Table 1: Calibration data of Levosalbutamol

Level	Concentration (µg/mL)	Peak area (mv)
Level -1	2.5	78483
Level -2	5	148882
Level -3	7.5	217916
Level -4	10	285322
Level -5	12.5	351698
Level -6	15	429246
Slope		**27768**
Intercept		**8759.6**
Correlation Coefficient		**0.9996**
Range: 2.5 to 15 µg/ml		

Table 2: Linearity study of Ipratropium bromide

Level	Concentration (µg/mL)	Peak area (mv)
Level -1	1	98376
Level -2	2	174136
Level -3	3	260504
Level -4	4	332347
Level -5	5	416044
Level -6	6	502146
Slope		**80469**
Intercept		**15617**
Correlation Coefficient		**0.9994**
Range: 1to 6 µg/ml		

Table 3: System precision

S. No.	Area of Levosalbutamol	Area of Ipratropium bromide
1.	**148862**	**174129**
2.	**148871**	**174196**
3.	**148887**	**174182**
4.	**148888**	**174210**
5.	**149001**	**174221**
6.	**148876**	**174197**
Mean	**148901**	**174189**
S.D	**1573.24**	**1905.525**
%RSD	**0.71**	**0.72**

Table 4: Method precision

S. No.	Area of Levosalbutamol	Area of Ipratropium bromide
1.	**147820**	**174821**
2.	**147982**	**173902**
3.	**147620**	**174122**
4.	**148732**	**174723**
5.	**147621**	**174920**
6	**147901**	**174832**
Mean	**147946**	**174553.4**
SD	**2198.087**	**2209.551**
%RSD	**0.89**	**0.750**

Table 5: Recovery study for Levosalbutamol

Level (%)	Fixed concentration (µg/ml)	Spiked concentration (µg/ml)	Amount found	% Recovery
50	5	2.5	2.46	99.47
100	5	5	5.03	100.30
150	5	7.5	7.51	100.16

Table 6: Recovery study for Ipratropium bromide

Level (%)	Fixed concentration (µg/ml)	Spiked concentration (µg/ml)	Amount Recovered	% Recovery
50	5	1	0.97	99.0
100	5	2	2.02	100.50
150	5	3	2.98	99.6

Table 7: Robustness study for Levosalbutamol

Condition	Mean area
Unaltered	148741
Wavelength at 243nm	147332
Wavelength at 247nm	146386
Mobile phase: • (Buffer(48):Methanol(52)) • (Buffer(52):Methanol(48))	 147632 149342

Table 8: Robustness study for Ipratropium bromide

Condition	Mean area
Unaltered	176543
Wavelength at 243nm	175438
Wavelength at 247nm	173972
Mobile phase: • (Buffer(48):Methanol(52)) • (Buffer(52):Methanol(48))	 176829 178764

Table 9: Analytical validation parameters

Parameter	Levosalbutamol	Ipratropium bromide
Linearity (µg/mL)	2.5-15	1-6
Slope	27768	80469
Intercept	8957.6`	15617
Correlation coefficient	0.9996	0.9994
LOD (µg/ml)	0.31	0.11
LOQ (µg/ml)	0.98	0.37
Theoretical Plates	6748	8218
Tailing Factor	1.12	1.23
Retention Time (min)	2.11	5.19

Table 10: Assay studies

Brand Name	Drug	Label Claim	Amount Found*	% Assay
DUOLIN	Levosalbutamol	5 mg	5.01mg	100.2
	Ipratropium bromide	2 mg	2.02 mg	101

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