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Research Article

RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF IPRATROPIUM BROMIDE AND LEVOSALBUTAMOL IN PHARMACEUTICAL METERED DOSE INHALERS

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ABSTRACT

An accurate, sensitive, precise, rapid and isocratic Reversed-Phase HPLC, (RP-HPLC) method for simultaneous estimation of Ipratropium bromide and Levosalbutamol in the bulk drug and in the pharmaceutical metered dose inhalers has been developed and validated. The best separation was achieved on a 250 mm × 4.6 mm i.d., 5 µm particle, Inertsil ODS 3V-RP C18 column with Acetonitrile as the organic modifier and Di-Potassium Hydrogen Phosphate [0.03M] in water with pH 3.2 adjusted with Ortho-Phosphoric Acid (0.1% v/v) in the proportion of [30:70 v/v] as mobile phase at a flow rate of 0.8 mL min^{-1}. UV detection was at 242 nm. Retention times were found to be 5.206 min. for Ipratropium bromide and 7.016 min. for Levosalbutamol.The response was a linear function of concentration over the range of 2.00 to 6.00 µg/ml, and 5.00 to 15.00 µg/ml respectively with correlation coefficient of 1.000 for Ipratropium Bromide and 0.994 for Levosalbutamol respectively. The percentage assay of Ipratropium Bromide and Levosalbutamol were found to be 99.87 %, and 101.42 % respectively. The Limit of Detection (LOD) for Ipratropium bromide and Levosalbutamol were found to be 1.27 µg/ml and 4.41µg/ml respectively. The Limit of Quantification (LOQ) for Ipratropium bromide and Levosalbutamol were found to be 3.81µg/ml and 13.23µg/ml respectively. The excipients present in the formulation were not interfered with the assay. The method is suitable for application in quality-control laboratories, because it is simple and rapid with good accuracy and precision.

Keywords: RP-HPLC, Ipratropium bromide, Levosalbutamol, Linearity, LOD & LOQ.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) refers to chronic bronchitis and Emphysema, which is a pair of two commonly co-existing diseases of the lungs in which the airways become narrowed[1]. COPD is also known as Chronic Obstructive Lung Disease (COLD), Chronic Obstructive Airway Disease (COAD), Chronic Airflow Limitation (CAL) and Chronic Obstructive Respiratory Disease (CORD). Important management strategies are smoking cessation, vaccinations, rehabilitation and drug therapy (often using inhalers). The combination of Ipratropium bromide and Levosalbutamol will help in targeting different aspects of COPD viz. bronchodilation through different mechanisms and the inflammations with inhaled steroids[2]. Ipratropium bromide is an anticholinergic drug[3] used for the treatment of COPD and acute asthma. It blocks the muscarinic acetylcholine receptors in the smooth muscles of the bronchi in the lungs, opening the bronchi. It is chemically [8-methyl-8-(1-methylethyl) - 8-azoniabicyclo [3.2.1] oct-3-yl] 3- hydroxy-2-phenyl-propanoate (Fig: 1). Levosalbutamol, is

the R-enantiomer of the short-acting β2-adrenergic receptor agonist salbutamol (Fig: 2). Literature survey reveals the determination of Ipratropium bromide[5,6] and Levosalbutamol[7,8] by using Liquid Chromatography in biological fluids like plasma and urine. Colorimetric methods, with laborious derivatization, have been reported for analysis of Ipratropium bromide in rotacaps[9]. In this article, a simple, easily available and reliable RP- HPLC method with UV-detection has been developed and validated for the simultaneous determination of Ipratropium bromide and Levosalbutamol concentrations in metered dose inhalers.

Experimental

Chemicals and Reagents

- Ipratropium bromide of 99%, (Molecular Weight: 412.37 g/mol) and Levosalbutamol of 99% (Molecular Weight: 239.31 g/mol) purity are acquired from Cipla Pharmaceuticals, Mumbai, India.
- Acetonitrile HPLC Grade from Rankem Fine chemicals of HPLC Grade.
- Potassium Phosphate (Dibasic, K_2HPO_4) [0.03M] from Rankem Fine Chemicals AR grade.
- Ortho-Phosphoric Acid, 85%, Quligens Fine chemicals and HPLC Grade water.

Chromatography Instrument

Quantitative HPLC was performed on liquid Chromatography, Waters separation 2996, PDA detector module equipped with automatic injector with the injection volume 20 µl, and 2693 pump. A RP Inertsil ODS 3V C-18 column (250x4.6 mm i.d; particle size 5 µm) was used. The HPLC system was equipped with Empower Software. The column was maintained at 40^o C and eluted under isocratic conditions over 14.0 min at a flow rate of 0.8 mL/min. Mobile phase consisted of Acetonitrile as the organic modifier and Di-Potassium Hydrogen Phosphate[0.03M] in water with pH 3.2 adjusted with Ortho-Phosphoric acid (0.1% v/v) in the proportion of [30:70 v/v.]. Before use, it was filtered through a 0.45 µm Nylon membrane filters and then degassed. UV detection was performed at 242 nm.

Preparation of the Primary Standard Drug solution

A standard stock solution of the drugs was prepared by dissolving 20 mg of Ipratropium bromide and 50 mg of Levosalbutamol in 10 ml volumetric flask containing 5 ml of diluent (50:50 v/v Acetonitrile: Water), sonicated for about 15 min and then made up to 10 ml with diluent to get the primary standard stock solution containing 2 mg /ml of Ipratropium bromide and 5 mg /ml of Levosalbutamol.

Preparation of Working Standard Drug Solution

1.0 ml of the above stock solution was taken in 100 ml volumetric flask and thereafter made up to 100 ml with diluent (50:50v/v Acetonitrile: Water) to get the working standard solution containing 20 µg /ml of Ipratropium bromide and 50 µg /ml of Levosalbutamol.

From the above working standard 1.0 ml, 1.5ml, 2.0ml,2.5ml & 3.0ml dilutions were made and transferred in 10 ml volumetric flask and thereafter made up to 10 ml with diluent (50:50v/v Acetonitrile: Water) to get the working standard solution containing 2-6µg/ml of Ipratropium bromide and 5-15 µg /ml of Levosalbutamol respectively.

Analysis of Pharmaceutical Metered Inhalers

Remove the pressurized container (Duolin Inhaler® MDI, Cipla; Each puff contains: Ipratropium bromide 20 µg /ml and Levosalbutamol 50 µg /ml are suspended in propellant HFA 227-q.s in net weight of contents equivalent to 21µg of Ipratropium Bromide and 60 µg of Levosalbutamol) from the actuator and remove all the labels and markings with suitable solvent. Dry the container, replace in its actuator, shake for about 30 seconds and prime the metered valve as follows. Discharge once for waste; wait for not less than 5 seconds and discharge again to waste. Remove the pressurized container from its actuator, clean the valve stem (internally and externally) and the valve ferrule by washing with a suitable solvent. Dry the complete valve assembly, using an air line fitted with an appropriate narrow jet to ensure that all solvent is removed from the inside of the valve stem. Place a tripod stainless steel base plate with a central circular indentation of 1.5 mm in diameter in a small vessel suitable for shaking and add 15 ml of diluent. The size of the vessel is such that when the pressurized inhalation is discharged in to 15 ml of diluent, discharge takes place not less than 25 mm below the surface of the solvent. Shake the pressurized container for about 30 seconds and place in inverted position in the vessel. Discharge 120 deliveries below the surface of the solvent actuating the valve at intervals of not less than 5 seconds, maintaining the

pressurized container in vertical plane and discharging the pressurized inhalation through the aperture of the base plate. Shake the pressurized container between each actuation of the valve. Shaking should be carried out without removing the pressurized container from inverted position in the vessel. Remove the pressurized container, wash it with mobile phase and combined solution and washings to 25 ml volumetric flask, and dilute up 100 ml to volume with diluent to get the concentration of 20 µg/ml of Ipratropium bromide and 50µg/ml of Levosalbutamol.

Linearity

Aliquots of working standard solution contains Ipratropium bromide and Levosalbutamol stock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of Ipratropium bromide and Levosalbutamol are in the range of 2.00 to 6.00 µg/ml and 5.00 to 15.00 µg/ml respectively (Table: 4). Each of these drug solutions (20µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 242 nm and a calibration graph was obtained by plotting peak areas versus concentration of Ipratropium bromide and Levosalbutamol.

Accuracy

Accuracy was evaluated in triplicate by addition of two different amounts of Ipratropium bromide and Levosalbutamol, to a previously analyzed sample and comparing the amounts of analytes recovered with the amounts added. The amounts added were equivalent to 80, 100, and 120% of the amount originally present. %Recovery and RSD (%) were calculated for amount added. From these data, it is obvious that the method is remarkably accurate, which ensures that this method produces reliable results (Table: 2).

Precision

Intra-day and Inter-day precision were evaluated by analyzing quality-control samples containing low, medium, and high concentrations of 80%, 100% and 120% For Intra-day variation, sets of five replicates of the three concentrations were analyzed on the same day; for Inter- day variation, five replicates were analyzed on three different days. The low value (≤1%) of RSD indicates the repeatability of the method (Table: 3).

Limits of Detection and Quantification

Limit of Detection (LOD) of the method was determined as the lowest concentrations of active pharmaceutical ingredients producing a signal-to-noise (S/N) ratio of about 3. The Limit of Quantification (LOQ) was determined as the lowest concentrations of active pharmaceutical ingredients capable of being quantified with acceptable accuracy and precision producing signal-to-noise (S/N) ratio of about 10.

Method Applicability

The present developed method was evaluated by applying to pharmaceutical metered dose aerosols for the estimation of Ipratropium bromide and Levosalbutamol by our research group.

Method

HPLC Method Development and Optimization

In response to lack of simple, reliable and easy-to-use method for the determination of Ipratropium bromide and Levosalbutamol concentrations in pharmaceutical matrices, an isocratic Reversed-Phase HPLC method was developed for quantification of above mentioned, active pharmaceutical ingredients. We examined several HPLC method variables with respect to their corresponding effects on the result of analysis. To optimize the chromatographic conditions, different combinations of Methanol-Water and Acetonitrile-Water and Acetonitrile- Di-Potassium Phosphate buffer were tested. Acetonitrile with Phosphate buffer system [pH 3.2] was preferred because it resulted in greater resolution of active pharmaceutical ingredients after several preliminary investigatory runs, compared with other mobile phases. The other parameters in this factorial design were temperature, flow rate, detection wavelength and volume of injection. Buffer molarity was changed and optimum buffer strength was selected as 0.03M on the basis of theoretical plate number. At 242 nm, UV responses of all three active pharmaceutical analytes were good and free form interferences. Under these conditions, the analyte peaks were well defined and free from tailing. Considering the whole body of the data obtained from this extensive study, the set of conditions indicated earlier in this article was selected for further validation. Typical chromatogram of Ipratropium bromide and Levosalbutamol has been shown in Figure: 3.

RESULTS AND DISCUSSION

Method Validation Tests

Recommended method validation characteristics including Method precision (RSD, %), Method accuracy (Recovery % and RSD, %), Linear range (Correlation Coefficient), and LOD & LOQ, were investigated.

Linearity

The plot of peak areas of each sample against respective concentrations were found to be linear in the range of 2.00-6.00µg/ml for Ipratropium Bromide and 5.00-15.00µg/ml for Levosalbutamol with Correlation Coefficient of 0.999 (Table: 4). Linear regression least square fit data obtained from the measurements are given in Table: 1. The respective linear regression equation being Y= 1083385.00x+57133.3508 for Ipratropium Bromide and Y= 4493398.85x+2297765.0215 for Levosalbutamol. The regression characteristics, such as slope, intercept, and %RSD were calculated for this method and given in Table: 1.These results show that there was an excellent correlation between peak areas and analyte concentration. (Fig: 4 & fig: 5).

Accuracy

Recovery of the individual substances at 80%, 100%, and 120% of specified concentrations were between 97.1% and 104.25%, which proves the accuracy of the method. From these data, RSD was always less than 1%, which indicates it is obvious that the method is remarkably accurate, produces reliable results (Table: 2).

Precision

The Intra-day and Inter-day variability or precision data are summarized in Table: 2. The low value (<1%) of RSD indicates the repeatability of the method. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs (Table: 3).

Robustness

Robustness was studied out to evaluate the effect of small but deliberate variations in the chromatographic conditions at three different levels, i.e. –2, 0, +2. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the detection wavelength by ±2 nm (240 nm to 244 nm), mobile phase buffer to Acetonitrile ratio (68:32 to 72:28, v/v), mobile phase pH by ±0.2 units (pH 3.0 to 3.4), and mobile phase flow rate by 0.8 mL min−1 (0.6 to 1.0 mL min−1) had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust. The results are shown in Table: 5.

Limit of Detection and Limit of Quantification

The Limit of Detection (LOD) for Ipratropium bromide and Levosalbutamol were found to be 1.27 mg/ml and 4.41 mg/ml respectively. The limit of Quantification (LOQ) for Ipratropium bromide and Levosalbutamol were found to be 3.81 mg/ml and 13.23 mg/ml respectively. These values reflect the high sensitivity of the method, which is of great importance in most studies and also indicating the method can be used for detection and quantification of analytes in a very wide concentration range.

Specificity

No evidence of signals, in the corresponding times of the chromatogram were monitored as a sign of potential interfering peaks, were found when the pharmaceutical metered dose inhalers were tested. Hence, this method can be used reliably for the estimation of respected active pharmaceutical ingredients in a variety of dosage forms.

CONCLUSION

A simple and easily available HPLC method was developed in this study for the quantification of Ipratropium bromide and Levosalbutamol in pharmaceutical matrices. The main advantages of this method are its considerably shorter run times, easy-to-use and its simplicity. All of these properties are very important in practice, particularly when a large number of samples are to be analyzed. The results of validation tests were, collectively, indicative for a method with a relatively wide linear range, acceptable precision and accuracy and practically reliable sensitivity. The method enables simple, selective, sensitive, and specific analysis Ipratropium bromide and Levosalbutamol and can be used for routine analysis in pharmaceutical quality control.

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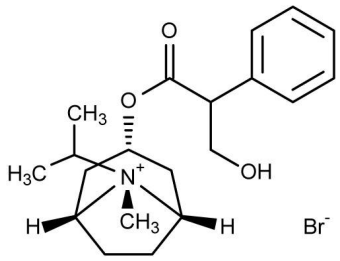

Fig. 1: Ipratropium bromide

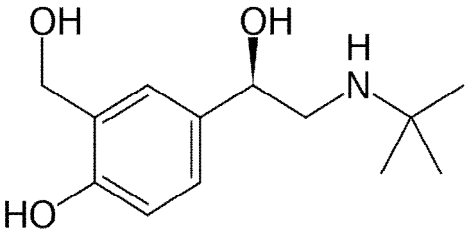

Fig. 2: Levosalbutamol

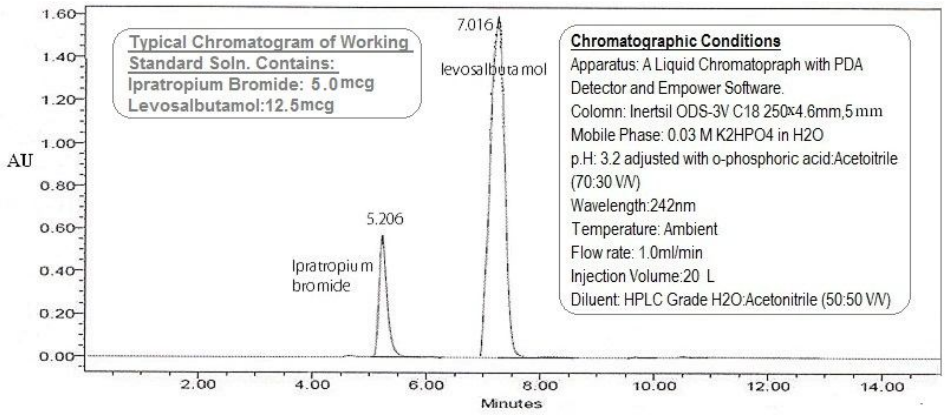

Fig. 3: Typical HPLC Chromatogram of Ipratropium Bromide and Levosalbutamol

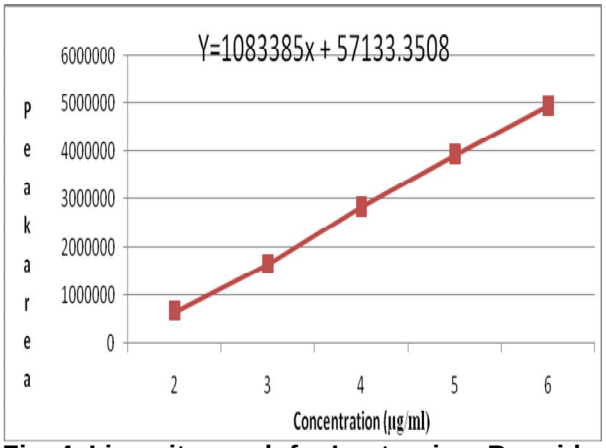

Fig. 4: Linearity graph for Ipratropium Bromide

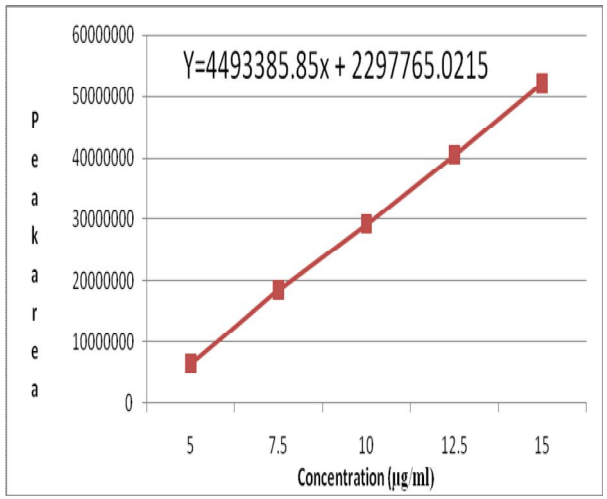

Fig. 5: Linearity graph of Lavosalbutamol

Table 1: Results from Regression Analysis and System Suitability data

Parameter	Ipratropium Bromide (µg)	Levosalbutamol (µg)
Retention Time (min)	5.206	7.016
Tailing Factor	1.67	1.24
Theoretical Plates	7629.29	9972.81
Resolution	0.94	1.83
Linear range in (µg/mL)	2-10	3-15
Limit of Detection (LOD) (µg/mL).	1.27	4.41
Limit of Quantification (LOQ) (µg/mL)	3.81	13.23
Slope(m)	766602	5309731.4
Intercept©	29930.11	184384.4
Correlation Coefficient (r)	1.000	0.994
Method Precision (RSD, %, n=6)	0.36	0.59
% of Assay	99.87	101.42

Table 2: Results of Recovery Accuracy Studies

Accuracy parameter	Spiked with 80% of the Working Std. Solution contains analytes in µg/mL		Spiked with 100% of the Working Std. Solution contains analytes in µg/mL		Spiked with 120% of the Working Std. Solution contains analytes in µg/mL	
	Ipratropium Bromide	Levosalbutamol	Ipratropium Bromide	Levosalbutamol	Ipratropium Bromide	Levosalbutamol
Amount added	4.7µg/mL	9.5µg/mL	5.7µg/mL	12.5 µg/mL	6.6µg/mL	14.96µg/mL
Amount Found	4.58µg/mL	9.390µg/mL	5.29µg/mL	12.2µg/mL	6.20µg/mL	14.87µg/mL
% Recovery	98.5	99.11	101.25	99.4	104.25	100.25
% RSD.	0.31	0.41	0.85	0.69	0.38	0.62

Table 3: Results of Recovery Precision Studies

Precision Parameter	80% of the Working Standard Solution contains analytes in µg/mL		100% of the Working Standard Solution contains analytes in µg/mL		120% of the Working Standard Solution contains analytes in µg/mL	
	Ipratropium Bromide (4.7µg/mL)	Levosalbutamol (9.5µg/mL)	Ipratropium Bromide (5.7µg/mL)	Levosalbutamol (12.5 µg/mL)	Ipratropium Bromide (6.6µg/mL)	Levosalbutamol (14.96µg/mL)
Intra-day Mean area ± SD	6447089±22366.4	65976000±243691	7523121.8±29460	77453813±216637	8578309±3206.4	88157110±57908
%.RSD	0.48	0.42	0.64	0.52	0.23	0.28
Inter-day Mean area ± SD	6462389±8163	66115095±88835.7	7401844.5±19380.4	76214655±272989	8574395±8330.6	88097823±222120
% RSD	0.35	0.37	0.53	0.60	0.31	0.45

Table 4: Summary of the Calibration data / Linearity studies

Concentration of Ipratropium Bromide (µg/mL)	Peak Area	Concentration of Levosalbutamol (µg/mL)	Peak Area
2.00	673768	5.00	6312272
3.00	1649095	7.50	18432329
4.00	2844280	10.00	29227236
5.00	3926159	12.50	40500237
6.00	4952161	15.00	52145492
Correlation Coefficient	1.000	------	0.994

Table 5: Results from testing of the Robustness of the method (n=3, 100% of the Working Standard Solution contains Ipratropium Bromide 7.2µg/mL and Levosalbutamol 10.8 µg/mL

Condition Studied in Robustness	Modification In OFAT analysis.	Mean Peak Areas ± S.D		% RSD		Mean Retention Time (in min) ± S.D	
		Ipratropium Bromide	Levosalbutamol	Ipratropium Bromide	Levosalbutamol	Ipratropium Bromide	Levosalbutamol
Detector Wavelength in (nm)	240	5572554± 2445.9	57494262 ±186637.6	0.56	1.18	5.221±0.84	7.059±1.18
	242	5522311± 12380.4	57455798± 222989.5	0.821	1.28	5.233±0.43	7.056±0.94
	244	5509629± 31681.8	57755569± 54908.3	0.789	0.921	5.217±0.42	7.057±0.58
Mobile Phase pH	3.0	5514140± 7821.9	57361218± 182324.6	0.921	0.825	5.236±0.65	7.034±0.32
	3.2	5502051± 5689.8	57250000± 239193.4	0.619	0.625	5.219±0.81	7.056±1.18
	3.4	5512170± 6437.5	57551700± 94432.3	0.527	0.825	5.234±0.94	7.063±1.27
Mobile Phase Composition in (v/v)	68:32	5510819± 18130.9	57241699± 96350.4	0.661	1.47	5.235±0.63	7.062±0.54
	70:30	5527875± 9995.2	57390457± 89445.8	0.982	1.19	5.228±1.04	7.068±0.76
	72:28	5519419± 2206.4	57318435± 268422.6	1.292	0.73	5.237±1.13	7.072±0.54
Flow rate in mL/minute.	0.6	5492663± 7830.6	56848412± 112360.2	0.654	0.58	5.229±0.76	7.083±0.69
	0.8	5537009± 3745.3	57726939± 162129.1	0.563	1.26	5.234±0.78	7.054±0.26
	1.0	5487356± 5689.7	56969661± 93318.2	0.76	1.58	5.228±0.65	6.877±0.82

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