JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF IPRATROPIUM BROMIDE AND LEVOSALBUTAMOL IN COMBINED DOSAGE FORMULATION.

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ABSTRACT:

A reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Ipratropium Bromide and Levosalbutamol in combined pharmaceutical formulation is developed. A Chromatographic separation carried out at 30^{o}c temperature on Agilent Zorbax SB-Aq (250 x 4.6 mm, 5 µ) column. Mobile phase consist of Acetonitrile: 0.1 % Perchloric acid (50:50, % v/v) with flow rate 0.8ml/min and the injection volume was 10 µl. The detection was done by Uv detector at 220nm and the run time was kept 7 min. Acetonitrile: Water (50:50, % v/v) used as a diluent. The retention time for Ipratropium Bromide and Levosalbutamol was at 4.14 and 3.03 respectively. The linearity range for Ipratropium Bromide and Levosalbutamol is 12-18 µg/ml and 36-54 µg/ml. The %RSD for accuracy was found to be less than 2%. High recovery of Ipratropium Bromide (98.8-100.1%) and Levosalbutamol (99.8-101.7%) indicates accuracy of the method. Validation parameters such as accuracy, linearity, precision, system suitability are considered as per International Conference on Harmonization guidelines. Based on all above parameters the proposed method was found to be simple, accurate, precise and reproducible and can be used for simultaneous estimation of Ipratropium Bromide and Levosalbutamol.

KEY WORDS: Ipratropium Bromide, Levosalbutamol, RP-HPLC

INTRODUCTION:

Ipratropium Bromide is chemically [8-methyl-8-(1-methylethyl)-8-azoniabicyclo [3.2.1] oct-3-yl] 3-hydroxy-2-phenyl-propanoate. Trade name for Ipratropium Bromide is Atrovent. It is a type of anticholinergic drug which opens the airways in the lungs. Ipratropium Bromide is mainly used in treatment of chronic obstructive lung disease and asthma. Ipratropium Bromide is only anticholinergic drug which is used for respiratory disease. It is used to prevent bronchoconstriction which results in dilation of airways. Acetylcholine (Ach) causes bronchial constriction and narrowing of air ways. Anticholinergic drug binds with acetylcholine receptor and prevent acetylcholine binding which results in bronchodilation.

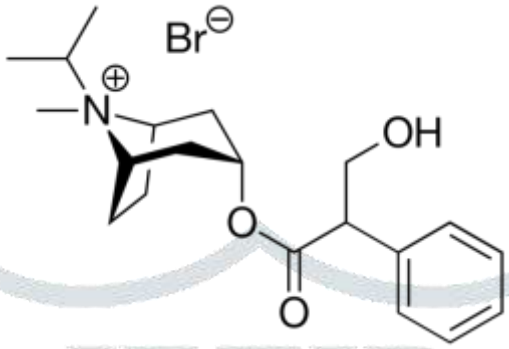

Figure 1: Structure of Ipratropium Bromide

Levosalbutamol is chemically 4-[(1R)-2- (tert-butylamino)-1-hydroxyethyl]-2- (hydroxymethyl) phenol. Levosalbutamol is primarily used as a short acting β_2 adrenergic receptor agonist. It is also known as levalbuterol. It is used in treatment of asthma and chronic obstructive pulmonary disease. Levosalbutamol binds with β_2 adrenergic receptor and causes activation of adenylate cyclase which results in increase in intracellular concentration of 3', 5'-cyclic adenosine monophosphate (cyclic AMP). Then the increased concentration of cyclic AMP activates protein kinase A which inhibits phosphorylation of myosin which decreases intracellular calcium concentration which results in muscle relaxation. Levosalbutamol causes relaxation of airways from trachea to terminal bronchioles.

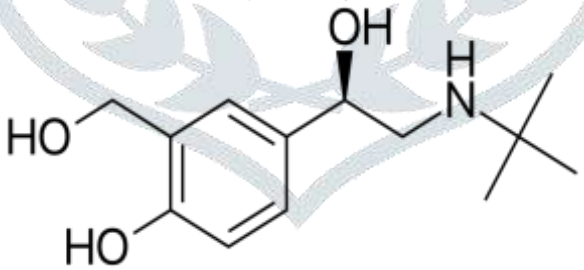

Figure 2: Structure of Levosalbutamol

Literature review declares that few spectroscopic methods are available for simultaneous estimation of ipratropium bromide and levosalbutamol. So new method was developed which is more easy and quick than previously published method for estimation of both drugs. The composition of mobile phase is adjusted to achieve highly accurate and specific results. The detection wavelength of 220nm was chosen in order to achieve a good resolution for quantitative determination of Ipratropium Bromide and Levosalbutamol in respules. So an attempt has been made to develop simple and economical method for simultaneous estimation of Ipratropium Bromide and Levosalbutamol in combined pharmaceutical formulation.

MATERIAL AND METHODS

Chemical and reagents:-

Analytical pure sample of Ipratropium Bromide and Levosalbutamol were received as a gift sample from Cipla Private Limited were used in the study. The combined pharmaceutical formulation used in this study was DEOLIN RESPULES. The labeled formulation contains Ipratropium Bromide and Levosalbutamol 500mcg/1.25 mg in 2.5 ml respules. The diluents used were of HPLC grade acetonitrile and water used in preparation of solutions.

Preparation of mobile phase:-

Mobile phase was prepared by mixing Acetonitrile and 0.1 % Perchloric acid (50:50, % v/v).

Apparatus and chromatographic conditions:-

Chromatographic separation is achieved by using Agilent Zorbax SB-Aq (250 x 4.6 mm, 5 µ) column. The elution was carried out at flow rate of 0.8ml/min using Acetonitrile and 0.1 % Perchloric acid (50:50, % v/v) as a mobile phase.

Standard Preparation:-

a. Standard Stock Solution-I (SSS-I):

i. Initially Prepare a Standard Stock Solution (SSS-I) of Ipratropium Bromide by adding 10 mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Ipratropium Bromide = 1000 µg/ml).

b. Standard Stock Solution-II (SSS-II):

i. Then prepare a Standard Stock Solution (SSS-II) of Levosalbutamol by adding 10 mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with Diluent. (Conc. of Levosalbutamol = 1000 µg/ml).

c. Standard Stock Solution-III (SSS-III)

i. Then add 1.5 ml of SSS-I & 4.5 ml SSS-II in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 150 µg/ml & Levosalbutamol = 450 µg/ml).

d. Further pipette out 1 ml of above solution in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 15 µg/ml & Levosalbutamol = 45 µg/ml).

Selection of Wavelength:-

The sample was scanned from 200-400 nm with PDA detector. The Wavelength selected for analysis was 220 nm on basis of appropriate intensity of both the peaks.

Table.1 Chromatographic conditions

column temperature	30^0c
flow rate	0.8ml/min
mobile phase	acetonitrile: 0.1 perchloric acid
Runtime	7 minutes
injection volume	10µl
Wavelength	220 nm
Diluent	acetonitrile and water (50:50,%v/v)
Column	agilent zorbax sb-aq
mobile phase ratio	50:50%v/v
RT of ipratropium bromide and levosalbutamol	4.14 min & 3.03 min

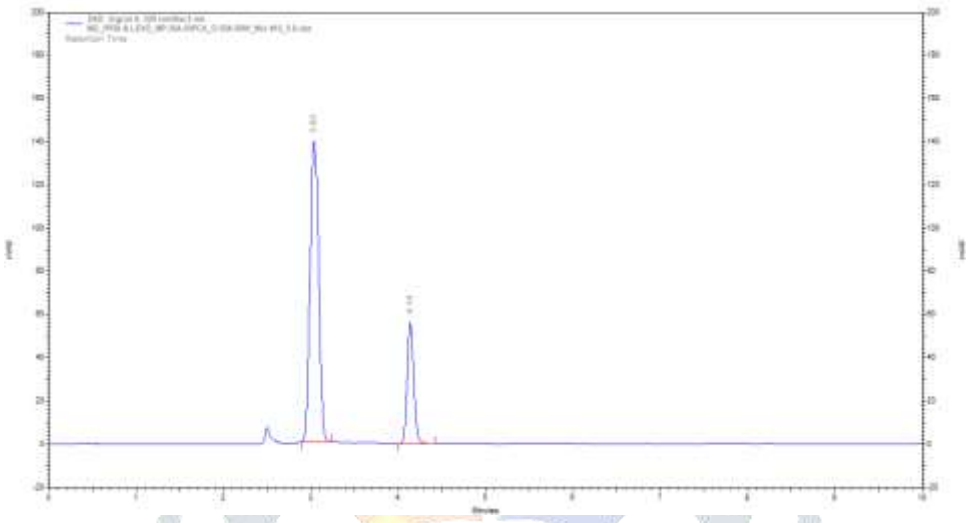

Figure.3: Chromatogram of standard mixture of Ipratropium Bromide & Levosalbutamol

Preparation of sample solution:-

i. 1 ml of Duolin Respules was taken to 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 150 µg/ml & Levosalbutamol = 450 µg/ml).
ii. Further pipette out 1 ml of above solution in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 15 µg/ml & Levosalbutamol = 45 µg/ml).

Assay:-

i. Individual samples of Ipratropium Bromide and Levosalbutamol were prepared of concentration 150 µg/ml and 450 µg/ml, respectively and peaks were for identified from Retention Time.
ii. Blank was injected to ensure there is no blank peak interfering with the main analyte peaks.
iii. 1 ml of Duolin Respules was taken to 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 150 µg/ml & Levosalbutamol = 450 µg/ml).
iv. Further pipette out 1 ml of above solution in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ipratropium Bromide = 15 µg/ml & Levosalbutamol = 45 µg/ml).

METHOD VALIDATION

Linearity:-

The developed method is validated in terms of linearity which is the ability to obtain test results which are directly related to concentrations of analyte in given sample. Linearity was studied by preparing varying concentration of Ipratropium Bromide and Levosalbutamol from standard stock solution.

i. 5 samples of varying concentrations ranging from 80-120% were made.
ii. The concentrations are given below in table 2.

Table 2: Linearity concentrations

% Level	Ipratropium Bromide Conc. (µg/ml)	Levosalbutamol Conc. (µg/ml)
80	12.0	36.0
90	13.5	40.5
100	15.0	45.0
110	16.5	49.5
120	18.0	54.0

Table 3: Linearity dilutions

Sr. No	Volume of Ipratropium Bromide stock solution to be taken(ml)	Volume of Levosalbutamol stock solution to be taken (ml)	Diluted to volume (ml)
1	0.8	0.8	10
2	0.9	0.9	10
3	1.0	1.0	10
4	1.1	1.1	10
5	1.2	1.2	10

Instrument Precision:-

Precision is used in explaining a statement related to set of results obtained when procedure is performed repeatedly to several samplings. Precision is performed by injecting six injections of single sample and then calculate standard deviation & % RSD.

If the obtained % RSD should be less than 2 then we can say that method is precise. The obtained % RSD for Ipratropium Bromide and Levosalbutamol is 0.12 & 0.46 respectively.

Accuracy:-

The term accuracy indicates the differences between obtained results and true results. The accuracy was determined by calculating recovery values of Ipratropium Bromide and Levosalbutamol by standard addition method. The standard addition method were carried out at different levels of 80- 120%. This is done by spiking a known amount of standard drug to a fixed amount of preanalysed sample solution. The average % recovery was observed. Samples were prepared of 80%, 100% and 120% concentration by spiking the same amount of concentration given above in table for both Ipratropium Bromide and Levosalbutamol.

Samples were injected in Triplicate to calculate % RSD. % recovery was also calculated which is given in table.

System Suitability:-

A single sample was prepared as described and 6 injections were made from same sample and checked for system suitability.

System suitability parameters are as below:

1. Retention Time,
2. Theoretical plates,
3. Asymmetry (Tailing factor)
4. Resolution.

Sensitivity:-

The sensitivity of method was determined in terms of LOD & LOQ.

Limit of detection (LOD) is a minimum amount of sample which can be detected and Limit of Quantification (LOQ) means smallest concentration of sample that can be determined with acceptable repeatability and accuracy.

It can be calculated for both drugs by using ANOVA technique.

Formula:

$$LOD = \frac{3.3 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

$$LOQ = \frac{10 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

The LOD & LOQ for Ipratropium Bromide was found to be 0.90 & 2.73µg/ml respectively & the LOD & LOQ for Levosalbutamol was found to be 1.96 & 5.95µg/ml respectively.

RESULT AND DISCUSSION

Assay:-

The % assay for Ipratropium Bromide was found to be 99.61% and for Levosalbutamol was 98.64%. Assay result given in below table 4.

Table 4: Results for assay of Ipratropium bromide and Levosalbutamol

Sample ID	Levosalbutamol		Ipratropium Bromide	
	Area	% Assay	Area	% Assay
Working Standard	201477	-	61427	-
Drug Product	200687	99.61	60589	98.64

Linearity of Ipratropium Bromide:-

Linearity study was done by plotting a graph of area v/s concentration. A different concentration of Ipratropium Bromide were prepared ranging from 12 µg/ml to 18 µg/ml is shown in below table**.** Linearity graph of Ipratropium Bromide is shown in Figure no.4.

Table 5: Linearity data of Ipratropium Bromide

% Level	Conc (µg/ml)	Area
80	12	48551
90	13.5	54980
100	15	61062
110	16.5	66369
120	18	72891

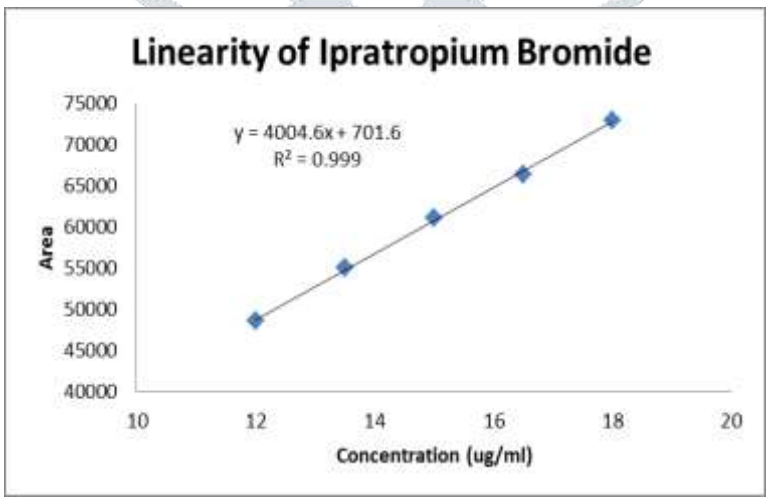

Figure4. Linearity graph of Ipratropium Bromide

Linearity of Levosalbutamol:-

Linearity study was done by plotting a graph of area v/s concentration. A different concentration of Ipratropium Bromide were prepared ranging from 36 µg/ml to 54 µg/ml is shown in below table. Linearity graph of Levosalbutamol is shown in Figure no.5.

Table 6: Linearity data of Levosalbutamol

% Level	Conc (µg/ml)	Area
80	36	164016
90	40.5	182778
100	45	201307
110	49.5	222258
120	54	241918

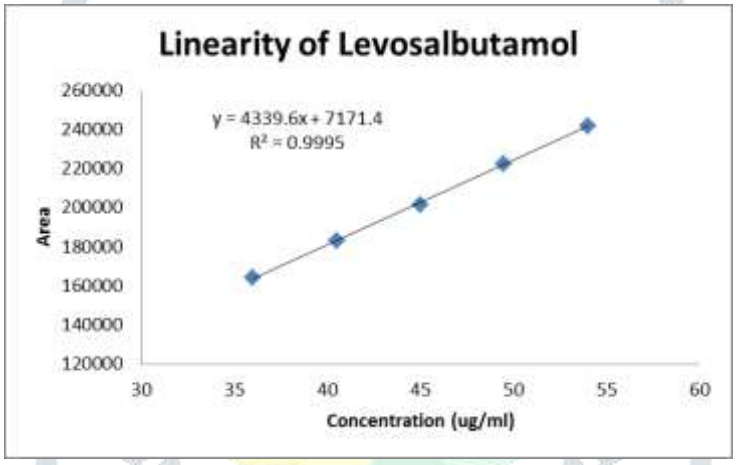

Figure.5:Linearity graph of Levosalbutamol

Precision of Ipratropium Bromide:-

The % RSD of Ipratropium Bromide was found to be 0.46 which is less than 2, so the proposed method was precise and the results are given in below table.

Table 7: Precision data of Ipratropium Bromide

Sample ID	Area
100% Rep 1	61062
100% Rep 2	61538
100% Rep 3	61257
100% Rep 4	61332
100% Rep 5	61478
100% Rep 6	61897
Average	61427
STDEV	285.2968
% RSD	0.46

Precision of Levosalbutamol:-

The % RSD of Levosalbutamol was found to be 0.12 which is less than 2, so the proposed method was precise and the results are given in below table

Table 8: Precision data of Levosalbutamol:-

Sample ID	Area
100% Rep 1	201307
100% Rep 2	201338
100% Rep 3	201227
100% Rep 4	201454
100% Rep 5	201887
100% Rep 6	201649
Average	201477
STDEV	248.5695
% RSD	0.12

Accuracy of Ipratropium Bromide:-

In accuracy study percentage recovery was calculated. The range of percentage recovery for Ipratropium Bromide is 98.80% to 98.13 %. The range of % RSD is 0.36% to 0.51%.

Table 9: Accuracy data for Ipratropium Bromide by RP-HPLC

Ipratropium Bromide			
Std Wt (mg)	% Purity	Stock Conc. (µg/ml)	Std Area
10	99.9	149.85	61427

% Level	Reps	Spiked Conc (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	% RSD
80	Rep 1	11.99	48551	11.84	98.80	0.36
	Rep 2	11.99	48264	11.77	98.21	
	Rep 3	11.99	48233	11.77	98.15	
100	Rep 1	14.99	61062	14.90	99.41	0.39
	Rep 2	14.99	61538	15.01	100.18	
	Rep 3	14.99	61257	14.94	99.72	
120	Rep 1	17.98	72891	17.78	98.89	0.51
	Rep 2	17.98	72193	17.61	97.94	
	Rep 3	17.98	72338	17.65	98.13	

Accuracy of Levosalbutamol:-

In accuracy study percentage recovery was calculated. The range of percentage recovery for Levosalbutamol is 101.76% to 99.96 %. The range of % RSD is 0.51% to 0.09%.

Table 10: Accuracy data for Levosalbutamol by RP-HPLC

Levosalbutamol			
Std Wt (mg)	**% Purity**	**Stock Conc. (µg/ml)**	**Std Area**
10	99.9	449.55	201477

% Level	Reps	Spiked Conc (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	% RSD
80	Rep 1	35.96	164016	36.60	101.76	0.51
	Rep 2	35.96	162338	36.22	100.72	
	Rep 3	35.96	163247	36.42	101.28	
100	Rep 1	44.96	201307	44.92	99.92	0.03
	Rep 2	44.96	201338	44.92	99.93	
	Rep 3	44.96	201227	44.90	99.88	
120	Rep 1	53.95	241918	53.98	100.06	0.09
	Rep 2	53.95	241497	53.88	99.89	
	Rep 3	53.95	241687	53.93	99.96	

System Suitability:-

Parameter of system suitability is Retention time, Therotical plates, Asymmetry (Tailing factor), Resolution is shown in table 11.

Table 11: system suitability parameter

Parameter	Ipratropium Bromide	Levosalbutamol
Retention time	4.14	3.03
Therotical plates	14620	4519
Asymmetry (Tailing factor)	1.13	1.11
Resolution	6.97	0.00

Sensitivity:-

LOD and LOQ of Ipratropium Bromide is 0.56µg/ml and 1.70µg/ml and Levosalbutamol is 14.08µg/ml and 42.65µg/ml.

Table 12: LOD & LOQ Data

Drugs	LOD µg/ml	LOQ µg/ml
Ipratropium Bromide	0.90	2.73
Levosalbutamol	1.96	5.95

CONCLUSION

Based on all above data concludes that the developed method is simple, rapid, precise and accurate and suitable for the simultaneous estimation of Ipratropium Bromide and Levosalbutamol in combined pharmaceutical formulation. The developed methods were validated as per ICH guidelines and were found to be within limit.

ACKNOWLEDGEMENT

I am very much thankful to Sahyadri College of Pharmacy, Methwade (Sangola), Maharashtra, for giving permission to carry out my work.

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