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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF FORMOTEROL AND TIOTROPIUM IN A CAPSULE DOSAGE FORM

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ABSTRACT

A simple, specific and accurate reverse phase high performance liquid chromatographic method was developed for the simultaneous determination of Formoterol and Tiotropium in capsule dosage forms. A phenomenex Supelco C-18, 5 µm column having 250 x 4.6 mm i.d. in gradient mode, with mobile phase containing 0.01 M potassium dihydrogen phosphate, pH 3.5:Acetonitrile(60:40),ortho phosphoric acid was used. The flow rate was 1.0 ml/ min and effluents were monitored at 230 nm. The retention times of Formoterol and Tiotropium were 3.7 and 4.7 min, respectively. The linearity for Formoterol and Tiotropium were in the range of 1.25-3.75 µg/ml and 2.25-6.75 µg/ml, respectively. The recoveries of Formoterol and Tiotropium were found to be in the range of 99.21 – 100.02%w/v and 98.59 – 100.56%w/v, respectively. The proposed method was validated and successfully applied to the estimation of Formoterol and Tiotropium in combined capsule dosage forms.

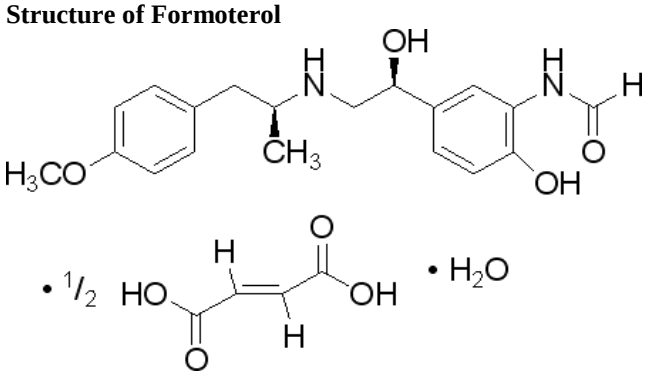
Keywords: Development and validation, RP-HPLC method, Formoterol, Tiotropium.

INTRODUCTION

Liquid chromatography is based upon the phenomenon that, under the same conditions, each component in a mixture interacts with its environment differently from other components [1]. Since HPLC is basically a separating technique, it is always used in conjunction with another analytical tool for quantitative and qualitative analysis. Advances in column technology, are high pressure pumping systems and sensitive detectors which have transformed liquid column chromatography into a high speed, high efficiency method of separation. This advanced technology is based upon the use of small bore (2.5 mm – internal diameter) columns and small particle size (3-50 µm) that allow fast equilibrium between stationary and mobile phases. This small particle column technology requires high pressure pumping system capable of delivering the mobile phase at high pressure, as much as 300 atmospheres, to achieve flow rates of several ml per minute. Since it is often necessary to use small amounts of analyte (usually less than 20 µg) with the column packing, sensitive detectors are needed [2].

Drug Profile
Formoterol

Structure of Formoterol



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CHEMISTRY

(R*, R*)-N-[2-Hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl] amino] ethyl] phenyl] form amide fumarate

a) Molecular formula: $C_{19}H_{24}N_2O_4 \cdot 0.5C_4H_4O_4 \cdot H_2O$

b) Molecular weight: 420.46.

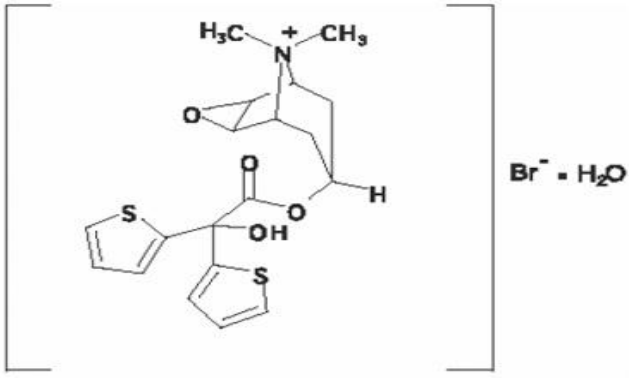
Appearance: Formoterol fumarate is white or slightly yellow powder

Solubility: Slightly soluble in water, Soluble in methanol, Slightly soluble in 2-propanol, Practically insoluble in Acetonitrile.

Category: Glucocorticoid

TIOTROPIUM BROMIDE

Structure



Chemistry

(1α, 2β, 4β, 5α, 7β–7–[(Hydroxydi–2–thienylacetyl) oxy]–9, 9–dimethyl–3–oxa–9–azoniatricyclo [3.3.1.$0^{2,4}$] nonane bromide monohydrate

a) Molecular formula: $C_{19}H_{22}NO_4S_2Br \cdot H_2O$.

b) Molecular weight: 490.4

Appearance: Tiotropium bromide is a white or yellowish white powder.

Solubility: It is sparingly soluble in water and soluble in methanol.

Category: Tiotropium bromide monohydrate is an anticholinergic with specificity for muscarinic receptors.

MATERIALS AND METHODS

Instrumentation:

1. Single pan balance (Metler toledo).
2. Control Dynamics PH Meter (Metler toledo).
3. HPLC-2695 Waters separations module.
4. DETECTOR- UV- Visible
5. Chromatographic data software – Empower.
6. Column – C_{18} -Supelco(250mm x 4.6mm), 5µm

Reagents and Chemicals

1. HPLC water
2. Acetonitrile (HPLC grade) - Merck specialty Pvt., Mumbai
3. Tri Ethyl Ammine - Merck
4. Orthophosporic Acid - Merck
5. Methanol - Merck

5.6

Reference Standards

FORMOTEROL - **Natco pharma ltd, hyderabad**.

% Purity - 100%

TIOTROPIUM BROMIDE - **Natco pharma ltd, Hyderabad**.

%Purity - 100%

PLACEBO - **Natco pharma ltd, Hyderabad**.

These two references standard were obtained as gift sample and the authenticity and purity of the sample was certified by the same.

METHOD DEVELOPMENT

Selection and standardization of mobile phase and column

Formoterol and Tiotropium bromide [3] are the combination, which are all ready exits in the market. The proposed method for estimation of Formoterol and Tiotropium bromide required adequate resolution of the two drug peaks in the chromatogram. Several solvent system and different columns were tried to obtain good optimum resolution [4-6].

Different combination of Methanol, water, Acetonitrile

1. Acetonitrile 100%
2. Acetonitrile: water (1:1)
3. Water 100%
4. Methanol: water (1:1)
5. Methanol (100%)

Peaks of Formoterol and Tiotropium bromide were all well resolved with the solvent system of 0.01M potassium dihydrogen phosphate buffer pH 3.5: Acitonitrile: (60::40).

Preparation of mobile phase

Preparation of Buffer

Dissolve 2.72g of Potassium di-hydrogen phosphate in 1000mL of purified water and add 2.0mL of Triethylamine and adjust the pH 3.5 (±0.05) with Orthophosphoric acid, through 0.22 µ nylon membrane filter and degas.

Preparation of Mobile Phase:

Solution A: Buffer pH3.5 (60%)

Solution B: Acetonitrile (40%)

Keep in gradient mode

Diluent: Solution A: Solution B (60:40)

Mix 600 ml of above buffer and 400 ml of Acetonitrile and degas by filtering through 0.22 membranes.

Diluent: Solution A: Solution B (60:40)

Determination of retention time

Preparation of Standard Stock solution A: (Formoterol Stock standard)

Accurately weigh and transfer about 50.0 mg of Formoterol fumarate dihydrate working standard

(Equivalent to 48.0mg of Formoterol fumarate) into a 200 mL volumetric flask. Add about 100 mL of Methanol, sonicate for 5 minutes, and make up with Methanol. Pipette 10 mL of above solution into 100 mL volumetric flask and dilute to volume with Diluent and mix.

Preparation of Standard Stock solution B: (Tiotropium Stock standard)

Accurately weigh about 46.0 mg of Tiotropium bromide monohydrate (Equivalent to about 36.0 mg of Tiotropium) Working standard and transfer into a 100 mL volumetric flask. Add about 50 mL of Methanol, sonicate for 5 minutes, and make up with Methanol. Pipette 10 mL of above solution into 100-mL volumetric flask and dilute to volume with Diluent and mix.

Preparation of Standard solution for Assay

Pipette 5 mL of Standard stock solution A and 5 mL of Standard stock solution B into 50 mL volumetric flask and dilute to volume with diluent and mix.

Method

Sample Preparation for Assay

Open and accurately weigh the contents of 10 intact capsules, and take that powder into a 50 mL volumetric flask. Add about 20 mL of Diluent and sonicate for 10minutes with occasional swirling to dissolve. Cool it and make up to the volume with diluent.

VALIDATION

Analytical method validation for assay of Formoterol fumarate and Tiotropium bromide shall be performed by carrying out the following typical analytical tests [7].

System Suitability

System suitability shall be done for chromatographic method by carrying out the Precision study with a minimum of 6 determinations at 100% level of standard concentration.

Acceptance criteria

RSD for areas of six replicates should be not more than 2.0 %.

The tailing factor for Formoterol fumarate and Tiotropium should be not more than 2.0. Number of Theoretical plates for Formoterol fumarate and Tiotropium should not be less than 2000.

Specificity

Specificity [8] is to validate the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present such as matrix components, impurities and degradation products. Evaluate the analytical method for Specificity by injecting the following solutions. Prepare diluent and inject into the HPLC system in triplicate. Prepare a sample with appropriate levels of excipients as a placebo sample and inject into the HPLC system in triplicate for all the dosage strengths. Prepare a solution of standard for assay (100% Conc) and inject into the HPLC system in triplicate. Prepare a solution of sample for assay (100% Conc) and inject into the HPLC system in triplicate.

Acceptance Criteria

It should not show any interference from the diluent and placebo at the retention time of Formoterol fumarate and Tiotropium.

The specificity of the method was evaluated by analyzing the sample solution spiked with excipients at appropriate levels that's the assay result is unaffected by the presence of extraneous materials.

Preparation of placebo (C)

Placebo is prepared by mixing all exicipients with out active ingredients.

Preparation of placebo stock solution

Standard preparation for Formoterol Fumarate: (Stock-A)

Standard weight 50.0mg Diluted to200mL with Methanol. Transferred 10mL above solution and diluted to100mL with Diluent.

Standard preparation for Tiotropium Bromide: (Stock-B)

Standard weight 46.0mg Diluted to100mL with Methanol. Transferred10mL of the above solution and diluted to100 mL with Diluent.

Preparation of mixed standard

Transferred 5mL of stock A & 5 mL of stock B in to 50mL with diluent.

Placebo preparation

Accurately weigh and transfer about 250mg of the placebo powder into a 50-mL volumetric flask. Add 25 mL of Diluent, mix and sonicate for 20 minutes. Cool and dilute to volume with diluent and mix. Inject the solution the chromatographic condition.

Linearity

Linearity [9] of an analytical procedure is to validate the ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. Linearity shall be established directly on the Formoterol fumarate and Tiotropium standard across the specified range with minimum 8 concentrations that are within the specified range. For linearity 50% to 150% (i.e. 50%, & 75%, 100%, 125%and 150%) of the test concentration (4.5 mcg/mL for Tiotropium and 2.5 mcg/mL of Formoterol fumarate) shall be considered as a minimum specified range of drug substance. Inject each linearity solution. Estimate the degree of linearity by calculating the correlation coefficient and Y-intercept/response at 100% of Working concentration and slope of the regression line should be calculated and reported. Establish a plot of data for analyte response Vs its concentration.

Acceptance Criteria

The squared correlation coefficient (r^2) of the linear regression should not be less than 0.99.

Y-intercept/response at 100% of working concentration x 100 ≤ 3.0%.

Preparation of Standard Stock solution A: (Formoterol Stock standard)

Accurately weigh and transfer about 50.0 mg of Formoterol fumarate dihydrate working standard (Equivalent to 48.0mg of Formoterol fumarate) into a 200 mL volumetric flask. Add about 100 mL of Methanol, sonicate for 5 minutes, and make up with Methanol. Pipette 10 mL of above solution into 100 mL volumetric flask and dilute to volume with Diluent and mix.

Preparation of Standard Stock solution B: (Tiotropium Stock standard)

Accurately weigh about 46.0 mg of Tiotropium bromide monohydrate (Equivalent to about 36.0 mg of Tiotropium) Working standard and transfer into a 100 mL volumetric flask. Add about 50 mL of Methanol, sonicate for 5 minutes, and make up with Methanol. Pipette 10 mL of above solution into 100-mL volumetric flask and dilute to volume with Diluent and mix.

Preparation of Standard solution for Assay

Pipette 5 mL of Standard stock solution A and 5 mL of Standard stock solution B into 50 mL volumetric flask and dilute to volume with Diluent and mix.

Precision

Precision of an analytical method is the degree of agreement among individual test result when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation or relative standard deviation [10].

Determination

The precision of an analytical method is determined by assaying sufficient number of sample and relative standard deviation is calculated. The precision of the instrument is determined by assaying the samples consecutively number of times and relative standard deviation is calculated.

Acceptance criteria: The relative standard deviation should be within 2%

System precision

Working standard solution

5ml of standard stock solution A 5ml of standard stock solution B was taken and transferred in to 50ml volumetric flask and dilute to 50ml to get a concentration 48mcg of Formoterol and 36mcg of Tiotropium.

Method: the system precision was evaluated by measuring the peak response of Formoterol and Tiotropium for six replicate injections of the mixed standard solution and chromatogram were recorded.

Method Precision

The method precision was determined by preparing the sample of single batch of Formoterol and Tiotropium formulation six times and six successive injections of 100µl of working sample solution A1 were injected and the chromatograms are recorded.

Accuracy

Accuracy [11] is to validate the closeness of test results obtained by the analytical procedure to the true value. The accuracy should be established across the specified range of the analytical method. Accuracy for the assay of drug products in which there is an extraction stage in the sample preparation is determined by applying the method in triplicate to samples or mixtures of excipients to which known amounts of analyte have been added at about 50%, 100% &150% of the nominal concentration. The accuracy is then calculated from the test results as the percentage of analyte recovered by the assay.

Acceptance criteria

The accuracy (recovery) for the average of triplicate in each concentration sample should be within 97.0 to 103.0%.

Robustness

Robustness [12] is to validate the analytical procedure capacity to remain unaffected by small but deliberate variation in method parameters and provides indication of its reliability during normal usage. Evaluate the analytical method robustness for the following typical variation from set procedure.

- Influence of variations of pH in mobile phase. Normal condition pH 3.5 (± 0.2 of the specified pH).
- Influence of variations of flow rate in mobile phase. Normal flow rate 1.0-mL/ min (± 0.2 of the specified flow rate).
- Influence of variations in wavelenth. Normal wavelength 230nm (± 5% of specified wavelength).

Acceptance criteria

The difference between average results when compared with the results obtained from specified method should be with in ±5.0 %. Difference between assay result obtained with centrifuged and filtered samples should be with in ± 2.0 %.

Solution stability

Sample and standard solutions should be tested over 24 hours period (Initial & 24 hours) under room temperature and normal lighting conditions, and potency of solutions should be determined by comparing the results against the freshly prepared standards.

Acceptance criteria

Acceptable stability difference relative to freshly prepared standard is ± 2.0% for standard and sample solutions.

Limit Of Detection (LOD) [13,14]

It is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated experimental condition. The detection limit is usually expressed as the concentration of analyte (e.g. parts per million). It is determined by based on the standard deviation of response and the slope.

Limit Of Quantitation (LOQ) [15]

The quantitation limit of an analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively with suitable precision and accuracy.
Based on the deviation of the response and the slope.

System Suitability Parameters

A solution of 25mcg/ml of formoterol and 46mcg/ml of tiotropium were prepared by diluting suitably with diluent and the same was injected in to the chromatograph and chromatogram was recorded.

Table 1. Chromatographic parameters

Instrument	HPLC-2695 Waters separations module
Column	C_{18} -Supelco(250mm x 4.6mm), 5µm
Wavelength	230 nm, UV-VIS Detection
Temperature	Ambient 30°C
Flow rate	1.0ml / min
Injection volume	100µl
Mobile phase	60:40,solutionA(pH 3.5 buffer):solution B (Acetonitrile)

Table 2. Specificity for Formoterol

S. No	Sample	Area obtained	% Content of drug w/v
1	Standard	612096	100.0%w/v
2	Standard + placebo	612096	100.0%w/v
3	Placebo	0	0

Table 3. Specificity for Tiotropium Bromide

S. No	Sample	Area obtained	% Content of drug w/v
1	Standard	687310	100.0%w/v
2	Standard + placebo	687310	100.0%w/v
3	Placebo	0	0

RESULT AND DISCUSSION

Table 4. Linearity data of Formoterol and Tiotropium

S. No	Linearity level at	Concentration (mcg/mL)		Response (Peak area)	
		Formoterol fumarate	Tiotropium Bromide	Formoterol fumarate	Tiotropium Bromide
1	50%	1.25	2.25	299446	347760
2	75%	1.875	3.375	443423	520944
3	100%	2.5	4.5	597229	696967
4	125%	3.0	5.4	731140	833255
5	150%	3.75	6.75	890217	1046636
The correlation coefficient (r^2)				0.9996	0.9999
%RSD				1.19	1.40
Y-intercept / response at 100% of working concentration x 100				2.30	1.55
Acceptance Criteria: The correlation coefficient (r^2) of the linear regression should not be less than 0.997 for 8 concentration points. Y-intercept / response at 100% of working concentration x 100 ≤ 3.0%.					
Conclusion: The Linearity test results meet / does not meet the acceptance criteria.					

Table 5. Precision data of the system

S. No	Area of Formoterol	Area of Tiotropium
1	612033	625482
2	613065	633210
3	616065	622350
4	610250	641509
5	608652	623515
6	606562	633095
Mean	611104.5	629860.2

S.D	3367.23	7392.06
%RSD	0.55	1.17

Table 6. System precision report for Formoterol & Tiotropium

Relative standard déviation	Formoterol	Tiotropium	Acceptance criteria
	0.55%	1.17%	2.0%

Table 7. Method precision of Formoterol

S. No.	Std Weight in mg	Area obtained	Assay value in mg	%Label claim w/v
1	50.1	590993	12.08	100.66
2	50.2	602151	12.34	102.83
3	50.1	591201	12.08	100.66
4	50.2	590223	12.09	100.75
5	50.1	588541	12.02	100.16
6	50.1	589621	12.05	100.41
Mean				100.9117
Standard deviation				0.880292
Relative standard deviation				0.872339

Table 8. Method precision of Tiotropium

S. No.	Std Weight in mg	Area obtained	Assay value in mg	%Label claim w/v
1	46.1	656507	18.28	101.55
2	46.1	650215	18.11	100.61
3	46.3	642141	17.95	99.72
4	46.0	647254	17.97	99.83
5	46.1	650114	18.1	100.55
6	46.1	649568	18.09	100.5
Mean				100.46
Standard deviation				0.600666
Relative standard deviation				0.597916

Table 9. Method precision report for Formoterol & Tiotropium

Relative standard déviation	Formoterol	Tiotropium	Acceptance criteria
	0.87%	0.59%	2.0%

Table 10. Recovery study data for Formoterol

S. No	Recovery	Area obtained	Average area	Amount recovered in mg	% Recovery w/w
1	50%	302839	304293.3	12.57	99.43
		303520			
		306521			
2	100%	606384	607440.7	25.19	99.24
		607358			
		608580			
3	150%	916320	918311	37.49	100.02
		918828			
		919785			

Table 11. Recovery study data for Tiotropium Bromide

S. No	Recovery	Area obtained	Average area	Amount recovered in mg	% Recovery w/w
1	50%	343128	342974.7	23.10	99.59
		341538			
		344258			

2	100%	692116 693562 694512	693396.7	45.60	100.89
3	150%	1042514 1032510 1021520	1032181	68.62	100.56

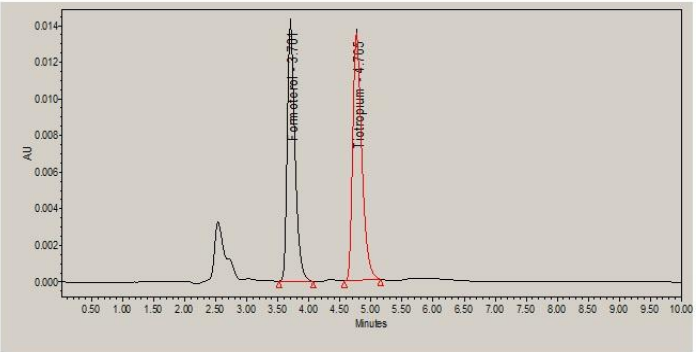
Table 12. System Suitability Report

System suitability factor	Formoterol	Tiotropium
Number theoretical plates	4482	4926
Resolution	4.1	
Tailing	1.45	1.52

Fig. 1. Assay of Formoterol and Tiotropium

Chromatogram

Sample name: specificity-std	Acq method set: formoterol_tiotropium assay
Injection volume:100µl	Processed channel descr:230 nm
Runtime:10mins	Column ID:RAD#097



S.no	Name	Retention time	Area	%Area	Height	Theoretical plates	Plate counts	S_N_Ratio
1.	Formoterol	3.701	612096	47.08	14090	4336	1.41	747.408
2.	Tiotropium	4.765	687310	52.92	13493	4338	1.42	715.723

Fig. 2. Linearity curve of Formoterol

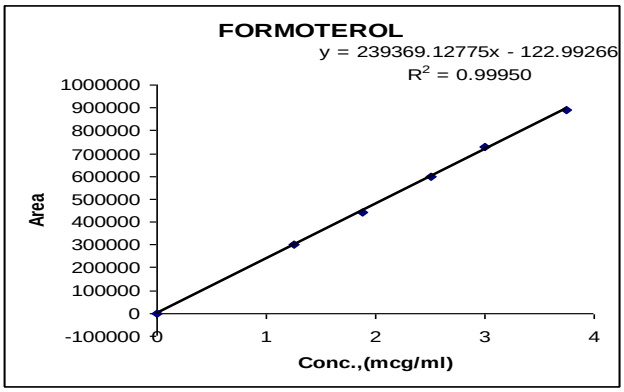
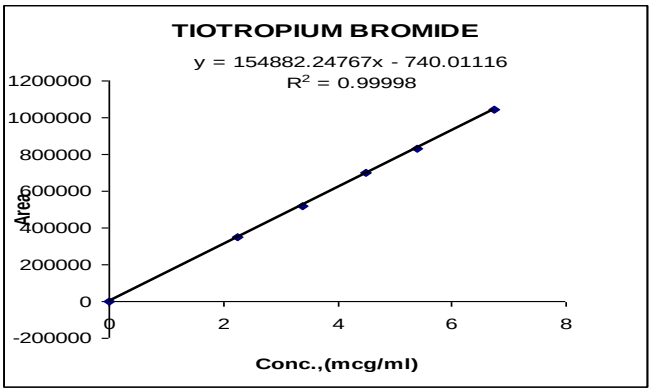


Fig. 3. Linearity curve of Tiotropium Bromide



As there is no official method for the estimation of Formoterol and Tiotropium Bromide in combination in pharmacopoeia. Combination of Formoterol and Tiotropium Bromide is increasingly finding use in treatment of Anti asthma and COPD disorders. Hence it was felt necessary to develop a sensitive method for simultaneous estimation of Formoterol and Tiotropium Bromide and literature review doesn't show any simultaneous method determination in this combined dosage formulation.

A method was developed with mobile phase system of buffer pH 3.5 and Acetonitrile (60:40) v/v with flow rate 1.0ml/min on C_{18} -Supelco(250mm x 4.6mm), 5µm particle size with UV detection 230nm gave a satisfactory chromatogram with Formoterol and Tiotropium Bromide of retention time 3.7 and 4.7 min. respectively. The net retention time for the two compounds in the reported method is about 10min.

Thus the present proposed system provides shorter analysis time and conserves mobile phase system. The method was validated based on British pharmacopoeia and ICH parameters. The parameters are accuracy, precision, linearity, specificity, Robustness, LOD, LOQ, and system suitability.

The specificity of the method was confirmed by injecting the placebo and placebo with spiked standard and observed that there was no interference due to placebo showed in Table 2 and 3.

The data regarding linearity of the two drugs are given in table 4 and calibration graph are shown in fig. 2 and 3. Linearity studies the specified range was determined for two drugs 1.25 –3.75mcg/ml for Formoterol and 2.25 – 6.75mcg/ml of tiotropium and linearity coefficient and percentage curve fitting was found to be 0.999 and 99.9 for Formoterol and 0.999 and 99.9 for tiotropium

The precision of the method was determined by replicate injections standard solution. The percentage of RSD of assay was to be in 0.87% for Formoterol and 0.59% for tiotropium which was with in the acceptance criteria of 2%. Thus the proposed method was found to be providing high degree of precision and reproducibility data are reported in table 7-9. The precision of the system was determined for replicate sample of Formoterol and tiotropium by multiple injections of a set of mixed solution of same concentration of Formoterol and tiotropium. The instrument response was found to be reproducible as found from %RSD of 0.55% for Formoterol and 1.17% of tiotropium that was well within the acceptance criteria of 2% (Table 6 to 9).

The validation of the proposed reverse phase HPLC method was further verified by recovery studies. The percentage recovery was found to be with in 99.24 – 100.02%w/v of Formoterol and 98.59 – 100.56%w/v of tiotropium. This serve a good index of accuracy and reproducibility of the proposed method are reported in table 10 and 11.

The limit of detection and limit of quantification of two drugs were calculated and found to be 0.01mcg/ml and 0.04mcg/ml for Formoterol and 0.04mcg/ml and 0.01mcg/ml for tiotropium.

The system suitability parameters were calculated to ascertain the suitability of the proposed method on the given system on C_{18} column and mobile phase of buffer pH 3.5 and Acetonitrile (60:40) ratio. The number of theoretical plates was found to be 4482 for Formoterol and 4926 for tiotropium (Table 12). The tailing factor for Formoterol is 1.45 and for tiotropium is 1.52 respectively.

The resolution of the method was good as found from the value of 4.1 indicating good and complete separation of the two components from each other with well defined base line.

CONCLUSION

A HPLC method is developed for the simultaneous estimation of formoterol and tiotropium combined dosage form using high performance chromatography. HPLC-Waters separations module 230 nm, UV-VIS Detection with and column C_{18} -Supelco(250mm x 4.6mm), 5µm. Injection volume of 100 µl is injected and eluted with the mobile phase of buffer pH 3.5: Acetonitrile in the ratio of 60:40 v/v, which is pumped at the flow rate 1.0ml and detected by UV detector at 230nm. The peaks of formoterol and tiotropium are found well separated at 3.7 and 4.7min respectively. The developed method is validated for various parameters as per ICH guidelines like accuracy, precision, linearity, Robustness, LOD, LOQ and specificity. The results obtained are with in the acceptance criteria. The proposed method is applied for determination of formoterol and tiotropium in marketed formulation. Hence the proposed method is found to be satisfactory and could be used for the routine analysis of formoterol and tiotropium in capsule dosage form.

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