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### RESEARCH ARTICLE

## A Novel Analytical Method Development and Validation of Tiotropium Bromide and Formoterol Fumarate Pharmaceutical Dosage Forms by using RP-HPLC Method

S. Priyanka\*<sup>1</sup>, M. Anuradha<sup>2</sup>, Kavita Waghray<sup>3</sup>

<sup>1</sup>Department of Pharmacy, University College of Technology, Osmania University, Hyderabad-500007, Telangana, India

<sup>2</sup>Pharmaceutical Analysis, Research Scholar, Department of Pharmacy, University College of Technology, Osmania University, Hyderabad-500007, Telangana, India.

<sup>3</sup>Professor, Dean of Pharmacy, Department of Pharmacy, University College of Technology, Osmania University, Hyderabad-500007, Telangana, India.

### ABSTRACT

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Tiotropium Bromide and Formoterol Fumarate was done by RP-HPLC Method. Separation was achieved under optimized chromatographic condition on stationary phase Inertsil C18 column (4.6 x 150mm, 5 $\mu$ m), the mobile phase consisting of Methanol : Phosphate Buffer (pH) in the ratio of 70:30 % v/v and pH was adjusted using orthophosphoric acid an isocratic elution was achieved at a flow rate 1.0 ml/min at an ambient temperature. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 1 ml/min. Calibration curves were linear with correlation coefficient not more than 0.999 over a concentration range of 100 to 500  $\mu$ g/ml of Tiotropium Bromide and 1 to 5 $\mu$ g/ml of Formoterol Fumarate respectively. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Tiotropium Bromide and Formoterol Fumarate. LOD and LOQ were found within limits. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, specific, precise, linear and rugged. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

**Keywords:** Inertsil C18, Tiotropium bromide and Formoterol fumarate, RP-HPLC

### ARTICLE INFO

#### Corresponding Author

S. Priyanka  
Department of Pharmacy,  
University College of Technology, Osmania University,  
Hyderabad-500007, Telangana, India  
MS-ID: IJCTPR3781



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### CONTENTS

1. Introduction. . . . .	26
2. Materials and Methods . . . . .	26
3. Results and Discussion. . . . .	28

4. Conclusion.....	28
5. Acknowledgement.....	29
6. Conflicts of Interest.....	29
7. References.....	29

## 1. Introduction

Tiotropium bromide is (1R,2R,4S,5S,7S)-7-[[2-hydroxy-2,2-bis (thiophen-2-yl)acetoxy]-9,9-dimethyl-3-oxa-9-azatricyclo[3.3.1.0<sup>2,4</sup>]nonan-9-ium bromide 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. Formoterol fumarate is N-[2-hydroxy-5-(1-hydroxy-2-[[1-(4-methoxyphenyl) propan-2-yl] amino] ethyl) phenyl] formamide hemifumarate is a long-acting, lipophilic, high-affinity 2 - selective agonist. Significant bronchodilation occurs within minutes of inhalation and may persist for up to 12 hrs. an advantage over many 2-selective agonists in settings such as nocturnal asthma [1-4]

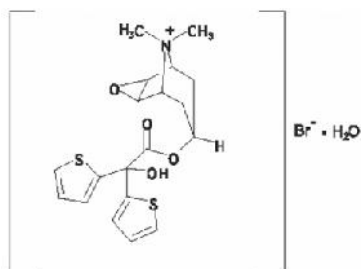


Fig. 1: Structure of Tiotropium Bromide

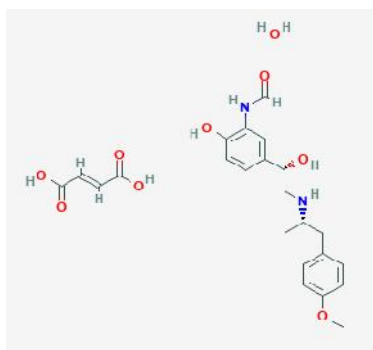


Fig. 2: Structure of Formoterol Fumarate

According to literature survey there is no RP-HPLC method available for determination of these analyte in combination by pharmacopoeias. Since there is no systematic method reported for Formoterol and Tiotropium in dry powder inhaler, a simple sensitive and precise method was developed and validated for Formoterol and Tiotropium by RP-HPLC. The developed methods were validated as per ICH guidelines.

## 2. Materials and Methods

**Materials:** Water, methanol and Ortho phosphoric Acid were of HPLC grade from E. Merck, India. Acetonitrile of HPLC Grade from Molychem, India. Potassium Dihydrogen Ortho Phosphate from FINER chemical LTD, International Journal of Current Trends in Pharmaceutical Research

India. Working standard of Formoterol and Tiotropium with potency 99.9% was obtained from Reddy's Pharmaceutical Ltd, India. Other chemical were analytical grade of above 99% purity. All volumetric ware was pre-calibrated by the manufacturer (borosil) and was of grade A Commercial capsules containing. Formoterol and Tiotropium (Tiomate Transcaps- Lupin) were procured from the local chemist shop.

### Instrumentation

The validated method utilized a WATERS 2695 separation module with PDA detector HPLC system with software – Empower with an isocratic elution technique at a flow rate 1ml/min at an ambient temperature. An Afcoset ER-200A balance was used for weighing purpose in this method. UV/VIS spectrophotometer LABINDIA UV 3000 was used for UV Determinations. [5-8]

### Chromatographic Conditions

The initially analysis was carried out with UV detection at 260 nm using a 20µl injection volume. Assay was performed using C18 reversed-phase column eluted with buffer pH:3.0: methanol (30:70 v/v) at a flow rate 1ml/min at an ambient temperature. The solvent were mixed, filter of 0.45 micron pore and degassed in ultrasonic bath prior to use. Dissolve 6.8 gm of sodium dihydrogen ortho-phosphate in 1000ml of triple distil HPLC grade water. Adjust pH 3.0 with orthophosphoric acid.

### Preparation of Standard Drug Solution

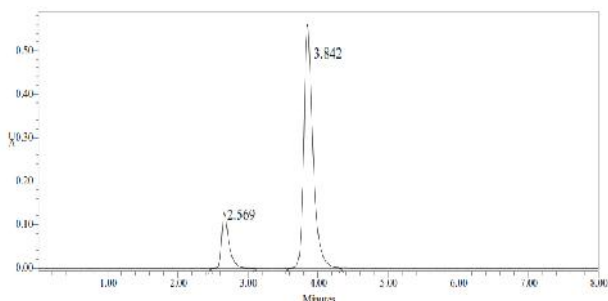
Weigh accurately about 10.0 mg of Tiotropium Bromide Monohydrate working standard and 10.0 mg of Formoterol Fumarate Dihydrate transfer in to 10ml and 100 ml volumetric flask respectively, add 7 ml of diluent and sonicate to dissolve with intermediate shaking. Make up the volume with diluent and mix.

### Mix Standard Solution

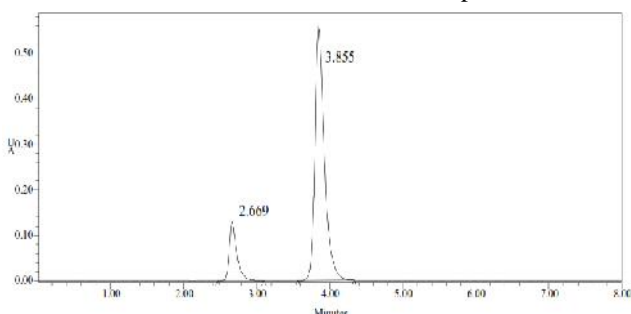
Dilute 3ml& 0.3ml of the above standard stock solutions respectively into a 10ml volumetric flask with diluent up to the mark and mix. Filter through 0.45µ Teflon filter (Concentration 0.3mg/ml of Tiotropium Bromide and 0.003mg/ml of Formoterol Fumarate) Figure 3.

### Preparation of Sample Solution

Transfer 10 opened capsule blend (equivalent to 50 mcg of Tiotropium and 0.5 mcg of formoterol fumarate dihydrate) into a 10 ml volumetric flask, take empty capsule shell into a dry beaker, add 20 ml of diluent into a beaker and rinse capsule shell and transfer the rinse into a 10 ml volumetric flask having blend. Sonicate the flask for 15 minutes, cool to room temperature and make up the volume with diluent to the mark and mix. Filter through 0.45µ Teflon filter and inject the filtrate. Dilute 3 ml of Tiotropium Bromide and Formoterol Fumarate of the above sample stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (Concentration 0.3mg/ml of Tiotropium Bromide and 0.003mg/ml of Formoterol Fumarate) Figure 4.



**Figure 3:** Chromatogram for Tiotropium Bromide and Formoterol Fumarate Standard Preparation



**Figure 4:** Chromatogram for Tiotropium Bromide and Formoterol Fumarate Sample Preparation

### Method Development

Optimized Chromatographic Conditions [11-12]

Instrument used : Waters HPLC with auto sampler and PDA detector.

Temperature : Ambient

Column : Inertsil ODS (150mm x 4.6mm x 5 $\mu$ m)

Buffer : Phosphate buffer

pH : 3.0

Mobile phase : 30% buffer: 70% Methanol

Flow rate : 1 ml per min

Wavelength : 260 nm

Injection volume : 20  $\mu$ l

Run time : 10min

### Method Validation [9-21]

**1. Specificity :** Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

#### 2. Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity studies should cover the range of 0-150% of the expected level of the analyte. The data is then processed using the method of least squares regression. The resulting plot, slope, intercept and correlation coefficient provide the desired information on linearity. ICH recommends that, for the establishment of linearity, a minimum of five concentrations should normally be used.

#### 3. Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy of the method was determined by %Recovery studies. To the formulation (pre analyzed

sample) and the reference standards of the drugs were added at the level of 50%, 100% & 150%. ICH documents recommend that accuracy should be assessed using a minimum of nine determinations covering the specified range of the procedure (i.e., three replicates of three concentrations) or using a minimum of six determinations at 100% of the test concentration.

#### 4. Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Procedure/method is applied to multiple sampling (5) of a homogenous sample

##### a. Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

b. Intermediate precision: Intermediate precision expresses under same conditions within same laboratory but different days, different analysts, different equipments and different reagents.

c. **Reproducibility:** Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology). Under different conditions like same different laboratory, different days (Intra-day if on one day, inter-day if on different days), different analysts, different equipments and different reagents

#### 5. Ruggedness:

The precision obtained when the assay is performed by multiple analysis, using multiple instruments, on multiple days, in one laboratory, different sources of reagents and multiple lots of columns should also be included in this study. Comparison of the reproducibility of test results to the precision of assay is the direct measure of Ruggedness

#### 6. Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range of the analytical procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy and linearity when applied to the samples containing analytes at the extremes of the range as well as within the range.

#### 7. Detection Limit (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

#### 8. Quantitation Limit (LOQ):

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Several approaches for determining the Quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

### 9. Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters may involve temperature ( $\pm 50^\circ\text{C}$ ), buffer pH ( $\pm 0.5$ ), ionic strength of buffers, level of additives to MP, flow rate ( $\pm 0.2\text{ml/min}$ ), wavelength ( $\pm 2\text{nm}$ ).

### 10. System Suitability:

It is essential for the assurance of the quality performance of chromatographic system. The accuracy and the precision of HPLC data collected, which begins with a well-behaved chromatographic system. Also used to ensure that proper concert of the selected chromatographic system.

## 3. Results and Discussion

### Specificity

Chromatograms of blank doesn't show any peak at the retention time of analyte peak. Result is shown in Figure 5.

### System Suitability

System suitability parameters such as number of theoretical plates, HETP and peak tailing are determined. The results obtained are shown in Table 1.

### Linearity

Tiotropium Bromide and Formoterol fumarate showed a linearity of response between 100 to 500 and 1 to  $5\mu\text{gml}^{-1}$  respectively. The correlation coefficient ( $r^2$ ) values were 0.999 and 0.999 respectively. These results are summarized in Table 2.

**Accuracy:** Recovery studies were performed to validate the accuracy of developed method. To pre-analyzed sample solution, a definite concentration of standard drug was added and recovery was studied. These results are summarized in Table 3.

### Precision and Intermediate Precision/Ruggedness

Six set of solution were analyzed in same day for repeatability and results were found within acceptable limits ( $\text{RSD} < 2$ ). Precision was performed by preparing six set of solution and check out reproducibility of result. The results for precision and ruggedness are shown in Table 4 indicating that acceptable precision was achieved for Formoterol and Tiotropium as revealed by relative standard deviation data ( $\text{RSD} < 2.0\%$  in all of the levels).

### Limit of Detection

Signal to noise ratio was 3 for LOD solution. So, the result obtained is within the limit.

### Limit of Quantification

Signal to noise ratio was 10 for LOQ solution. So, the result obtained is within the limit

### Robustness

It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed and System suitability parameters were found to be within acceptable limits. The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust. Results of analysis were summarized in Table 5 and 6.

### Tablet Analysis

Content of Formoterol and Tiotropium found in the Capsules by the proposed method are shown in Table 7. The low values of R.S.D. indicate that the method is precise and accurate.

## 4. Conclusion

The proposed method gives good resolution between Formoterol and Tiotropium within short analysis time (10 min). The method is very simple, specific, precise and economic and no complicated sample preparation is needed. High percent of recovery shows the method is free from interference of excipients present in the formulations. The proposed HPLC method is precise and accurate for the simultaneous determination of Formoterol and Tiotropium in combined dosage Form (Tiomate Transcaps Dry Powder Inhaler) as per ICH guidelines.

**Table 1:** Results of system suitability parameters for Tiotropium Bromide and Formoterol Fumarate

Sl.No	Parameters Name	Retention time(min)	Area ( $\mu\text{V sec}$ )	Height ( $\mu\text{V}$ )	USP resolution	USP tailing	USP plate count
1	Tiotropium Bromide	2	124505	213642		1.2	4673.4
2	Formoterol Fumarate	3	1308495	154566	6.0	1.3	6090.3

**Table 2:** Analytical performance parameters-Linearity of Tiotropium Bromide and Formoterol Fumarate

Parameters	Tiotropium Bromide	Formoterol Fumarate
Slope (m)	66574	12
Intercept (c)	53592	50
Correlation coefficient ( $R^2$ )	0.999	0.

**Table 3: Accuracy (Recovery)**

%Concentration	Tiotropium Bromide			Formoterol Fumarate		
	50%	100%	150%	50%	100%	150%
Area	656659.5	1304258	1854608	65800	124353	177940
Amount Added (mg)	5.0	10.0	14.4	5.3	10	14.2
Amount Found (mg)	5.036	10.003	14.224	5.34	10.10	14.45
% Recovery	100.7%	100.0%	98.78%	100.8%	100.01%	99.68%
Mean Recovery	99.84%			100.51%		

**Table 4: Results of method precision and Intermediate Precision/Ruggedness**

	PRECISION		RUGGEDNESS	
	Tiotropium Bromide	Formoterol Fumarate	Tiotropium Bromide	Formoterol Fumarate
	Area	Area	Area	Area
Injection-1	1302729	123149	1300148	122487
Injection-2	1302947	123766	1304520	122626
Injection-3	1303236	124271	1305937	122632
Injection-4	1303977	124691	1306476	122702
Injection-5	1309759	124956	130871	122962
Average	1304529.8	124162.7	1305070.2	122681.8
Standard Deviation	2961.1	725.6	3061.8	174.8
%RS	0.2	0.6	0.2	0.1

**Table 5: Robustness-Flow Rate (ml/min) data**

S. No	Flow Rate (ml/min)	System Suitability Results Tiotropium Bromide		System Suitability Results Formoterol Fumarate	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	0.	5339.9	1.4	7063.3	1.3
2	0.	4673.4	1.3	6090.3	1.2
3	1.	5216.0	1.4	6998.0	1.3

**Table 6: Robustness-Change in Organic Composition in the Mobile Phase**

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results Tiotropium Bromide		System Suitability Results Formoterol Fumarate	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	10% less	4508.4	1.3	6387.7	1.2
2	*Actual	4673.4	1.4	6090.3	1.2
3	10% more	4318.1	1.3	6232.5	1.32

**Table 7: Results of %RSD**

S.No	Parameter	Tiotropium Bromide	Formoterol Fumarate
1	SD	3011.45	450.2
2	%RSD	0.2	0.8

## 5. Acknowledgement

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## 6. Conflicts of Interest

The authors declare no conflicts of interest.

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