QUANTITATIVE DETERMINATION OF RELATED SUBSTANCES IN FORMOTEROL FUMARATE AND TIOTROPIUM IN TIOMATE TRANSCAPS® DRY POWDER INHALER

TIOMATE TRANSCAPS® KURU TOZ INHALERDE FORMOTEROL FUMARAT VE TIOTROPIUM'DAKI ILGILI MADDELERIN KANTITATIF TAYINI

Running Title: Estimation of related substances in Tiomate transcaps® DPI

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Abstract
Tiotropium (TIO) and Formoterol fumarate (FF) combination in dry powder inhaler dosage form used in the treatment of asthma, bronchospasm, chronic bronchitis, emphysema and chronic obstructive pulmonary diseases (COPD). Aim to develop an analytical method for the estimation of emerging and advancing dry powder inhaler combination towards enhanced therapeutics for the estimation of related substances but for this it is foremost to have a sensitive, simple, robust and validated method therefore, a new RP-HPLC method has been developed for the determination of related substances in Formoterol fumarate and Tiotropium in Formoterol fumarate dihydrate and Tiotropium bromide dry powder for inhalation. The chromatographic separation utilises an gradient elution in which buffer solution pH 3.2 used as mobile phase A and acetonitrile as mobile phase B at 1.0 mL min−1 flow rate, 30°C column temperature, and PDA detector at wavelength 240nm and Hypersil BDS C18 column (250 x 4.6mm, 5µm). Being validated in accordance with ICH guidelines, this method provides a safer and easier solution for QC testing and Stability studies for the related substances test.

Keywords: Dry powder inhaler, forced degradation study, LOD and LOQ, method validation, related substances

Öz
Asthma, bronkopazm, kronik bronşit, amfizem ve kronik obstrüktif akciğer hastahıklarının (KOAH) tedavisinde kullanılan kuru toz inhaler dozumu Tiotropium (TIO) ve Formoterol fumarat (FF) kombinasyonu. İlgili
maddelerin tahmini için geliştirilmiş terapötiklere doğru ortaya çıkan ve ilerleyen kuru toz inhaler kombinasyonunun tahmini için analitik bir yöntem geliştirme hedefleyin, ancak bunun için en önemi hassas, basit, sağlam ve onaylanmış bir yöntem olmak üzere, bu nedenle yeni bir RP-HPLC Formoterol fumarat ve Formoterol fumarat dihidrat içindeki Tiotropium ve inhalasyon için Tiotropium bromide kuru tozda ilgili maddelerin belirlenmesi için yöntem geliştirilmiştir. Kromatografik ayırma, tampon çözeltisi pH 3.2'nin mobil faz A olarak ve asetonitrilin 1.0 mL.min⁻¹ akış hızında, 30°C kolon sıcaklığında ve 240nm dalga boyundan PDA detektöründe ve Hypersil BDS C18 kolonundaki (250) mobil faz B olarak kullanıldığı bir gradyan elüsyonu kullanılarak X 4,6 mm, 5 um). ICH yönergelerine göre doğrulanmış olan bu yöntem, ilgili maddeler testi için QC testi ve Stabilite çalışmaları için daha güvenli ve daha kolay bir çözüm sağlar.

Introduction
Chronic bronchitis and Emphysema are the two existing lung diseases in which the airway become narrow and is collectively named as chronic obstructive pulmonary disease (COPD). [1] Essential management approaches are stopping smoking habit, vaccinations, rehabilitation and treatment by using inhalers. The combination of FF and TIO is used in targeting various characteristics of COPD as like bronchodilation and the inflammations. [1, 2] Formoterol fumarate dihydrate (FF) is a directly acting sympathomimetic with beta-adrenoceptor stimulant activity. FF is prescribed for its long acting beta 2 agonist effect in the treatment of airway obstruction, asthma and chronic obstructive pulmonary diseases. [3] The pharmacological effect of beta 2 agonist is to stimulate intracellular adenyl cyclase enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3',5'-adenosine monophosphate (Cyclic AMP). Increased cyclic AMP levels causes relaxation in the release of immediate hypersensitivity mediators from mast cells. Chemically, it is N-2-hydroxy-5-(1RS)-1-hydroxy-2-(1RS)-2(4methoxyphenyl)1methylethylaminoethyl phenyl formamide(E)-butenedioatedihydrate with molecular formula C₄₂H₅₂N₄O₁₂·2H₂O and molecular weight of 840.92. [1-2] Tiotropium bromide monohydrate (TIO) is an anticholinergic, antimuscarinic bronchodilator used in the airway obstruction, chronic obstructive pulmonary disease conditions. [1-3] Tiotropium shows its pharmacological effects by inhibiting M3 receptors present at the smooth muscle which leads to bronchodilation. Chemically it is (1R,2R,4S,5S,7s)-7-(2-hydroxy-2,2-dithiophen-2-ylacetyl)oxy-9,9-dimethyl-3-oxa-9-azoniatriacyclo3.3.1.02,4nonanebromidemonohydrate with molecular formula C₁₉H₂₂BrNO₄S₂·H₂O and molecular weight of 490.40.[1]

Complete literature survey reveals that TIO is determined by spectrophotometric method. [4] TIO in bulk and dry powder inhalation form is determined by HPTLC [5]. Methods are available to determine TIO and its related substances by HPLC. [6] For the biological estimation of TIO in human plasma; three methods illustrated.[7-9] Estimation of FF in various pharmaceutical dosage form by spectrophotometry with charge transfer complexation technique [10, 11], Q absorbance ratio and solving simultaneous equation [12], and zero order spectrophotometric method and area under curve (AUC) technique [13]. FF also estimated in combination with other drug moieties by thin layer chromatography (TLC) densitometry methods [14–17]. FF also estimated in combination with other drug moieties in by HPLC [14, 17–18], also in plasma, urine and biological samples [25, 26]. TIO has been determined with either FF [27–29] or clenbuterol or olodaterol [30-33] in various dosage forms by HPLC methods but the main focus was found to be on a single drug compound. In FF the Hydrazine hydrate content is determined by GC-MS method [34]; Moreover no related substances analytical method available in any of the pharmacopoeias. To the best of the author’s knowledge, no simple, sensitive and robust related substances analytical method which focused on both the drug moieties reported till now for the simultaneous evaluation of TIO and FF in dry powder inhaler dosage form and validated according to ICH guidelines. [35] The proposed validated RP-HPLC method can therefore be applied for simultaneous evaluation of TIO and FF QC testing and stability studies for the determination of related substances. To perform this study Tiomate transcaps® dry powder inhaler manufactured by Lupin Ltd. India is used.

MATERIAL AND METHODS
Instrumentation
The Dionex HPLC system consist of dionex ultimate 3000 UHPLC system equipped with quaternary gradient pump dionex ultimate 3000 pumps, dionex ultimate 3000 auto sampler, dionex ultimate 3000 column compartment and a dionex ultimate 3000 UV-Photo Diode Array detector. Separation and quantitation were carried out using a C18 Hypersil BDS column (250mm x 4.6mm, 5µm) Chromelion 7.2 SR5 software used for data acquisition.

Chemicals and Reagents
Pharmaceutical respiratory grade TIO was provided and qualified by Vamsi lab Ltd (India) as such assay was found to be 101.79%. Pharmaceutical grade FF was provided and qualified by Vamsi lab Ltd (India) as such assay was found to be 100.12%. HPLC grade acetonitrile (Rankem), Milli-Q water (Milli-Q® CLX 7000), sodium dihydrogen phosphate monohydrate, triethylamine, orthophosphoric acid (Rankem), 0.45 μm Buffer filter (mdi) was used.

**Chromatographic conditions**

The chromatographic separation utilises a gradient elution in which buffer consists of 1.38 gm of sodium dihydrogen phosphate monohydrate in 1000 mL of water, add 2mL of triethylamine, adjust pH 3.2 with dilute orthophosphoric acid, filter and degas through 0.45 μm filter. Mobile phase A is buffer solution pH 3.2 and mobile phase B is acetonitrile 1.0 mL min⁻¹ flow rate and BDS Hypersil C18 (250 × 4.6 mm, 5 μm). Diluent consists of a mixture of buffer pH 3.2 and Acetonitrile in the ratio of 70:30%v/v. Analysis was carried out at 30º C column temperature and PDA detector at wavelength 240nm for both TIO and FF. The injection volume was 100 µL and run time was 50 min. The Retention time of FF and TIO was found to be at 7.8 and 10.3 min respectively.

Gradient program is as follows:

| Time (minutes) | % Mobile phase : A (mL/min) | % Mobile phase : B (mL/min) |
|----------------|----------------------------|----------------------------|
| 0              | 80                         | 20                         |
| 30             | 60                         | 40                         |
| 40             | 30                         | 70                         |
| 45             | 30                         | 70                         |
| 50             | 80                         | 20                         |

**Standard Preparation**

**TIO standard stock solution**

Standard solutions of TIO were prepared by taking 36mg of TIO separately in each 100 mL volumetric flask, added 70mL of diluent sonicate to dissolve and make volume with diluent and mix. Further dilute 5mL of this solution to 100mL with the diluent.

**FF standard stock solution**

Standard solutions of FF were prepared by taking 24mg of FF separately in each 50 mL volumetric flask, added 35mL of diluent sonicate to dissolve and make volume with diluent and mix. Further dilute 1mL of this solution to 100mL with the diluent.

**Mix Standard Solution**

Pipette out 5mL of TIO standard stock solution and 10mL of FF standard stock solution to 100mL with diluent.

**Sample Preparation**

Tiomate transcaps® (Lupin LTD.) preparation, carefully open and collect the sample powder equivalent to 0.72 mg of TIO in to 10mL volumetric flask, added about 7mL diluent sonicate for 15 minutes with intermediate shaking, cool and dilute to volume with diluent and mix well and filter the solution through 0.45 µm filter by discarding the first few mL of the filtrate and use.

**Procedure**

Separately inject equal volume of the diluent, placebo solution, standard and sample solutions, record the peak responses. Disregard any peaks area due to diluent, formoterol fumarate and placebo solution in sample solution. Calculate the % of each impurity present in sample solution by following formulae,

\[
\text{Similarity factor} = \frac{\text{Area of Standard -1}}{\text{Area of Standard -2}} \times \frac{\text{Wt. of Standard -2}}{\text{Wt. of Standard -1}} \times 100
\]
% Impurity = \frac{AT}{AS} \times \frac{Wt.\,std}{100} \times 5 \times \frac{Wt.\,spl}{100} \times \frac{P}{100} \times \frac{Avg.\,Wt}{L.C.} \times \frac{392.5}{490.4} \times \frac{100}{1000}

where,

AT : Area of each impurity in sample solution
As : Area of standard solution 1
Wt. std. : Weight of standard in mg
Wt. spl. : Weight of sample in mg
Avg. Wt : Average weight of net content in mg
L.C. : Label Claim in mcg
P : Potency of standard
392.5 : Molecular weight of Tiotropium
490.4 : Molecular weight of Tiotropium bromide monohydrate

Analytical method development and optimization

The milli-Q water in different proportions of methanol and acetonitrile tried in both isocratic and gradient elution as well as by using various C8 and C18 columns but no proper separations was achieved. Different proportions of potassium and sodium salt buffers (10mMol to 30mMol) with methanol and/or acetonitrile were used in various proportions in both isocratic and gradient elution pattern but no proper peak shape, tailing factor and theoretical plates of TIO and FF was observed; also resolution between TIO and FF was not good. Various ranges of pH were tried from pH 2.5 to pH 6.5 and found that the best results was obtained with sodium dihydrogen phosphate monohydrate buffer pH 3.2 and acetonitrile 1.0 mL min−1 flow rate and BDS Hypersil C18 (250 × 4.6 mm, 5 μm). Diluent consists of a mixture of buffer pH 3.2 and Acetonitrile in the ratio of 70:30 %v/v. Analysis was carried out at 30º C column temperature and PDA detector at wavelength 240nm for both TIO and FF. The injection volume was 100 μL and run time was 50 min. The Retention time of FF and TIO was found to be at 7.8 and 10.3 min respectively.

Analytical method validation parameters

The comprehensive and systematic method validation was carried out as per ICH guidelines. The analytical method was validated for system suitability, system precision, method precision, intermediate precision, ruggedness, specificity, selectivity, forced degradation, linearity & range, accuracy, LOD & LOQ determination, precision at LOQ level, filter validation, robustness (change in chromatographic conditions) and stability of an analytical solution.

System suitability and System precision were determined by injecting two and six replicate injections of the standard solutions respectively. The responses of peaks were recorded.

In LOD and LOQ determination, a series of standard preparations of FF and TIO standard over the range starting from 1% to at least 50% of standard concentration were prepared. Plotted linearity graph of average area at each level against the concentration (ppm) and determine the correlation coefficient, slope and intercept of analyte for LOQ determination. The concentrations for limit of detection & limit of quantification from linearity study were determined.

Method precision may be defined as the precision of an analytical procedure express the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. In method precision six samples were prepared as per the analytical method representing a single batch; % impurities of these samples were determined for both the analytes and the analytical method precision was assessed by the % RSD.

Intermediate precision (Ruggedness) expresses ability of an analytical method to remain unaffected and produce reliable results within laboratory variation such as different days, different equipment, different analysts etc. Six samples were prepared as per the analytical method representing the same batch used for method precision. % impurities of these samples were determined for both the analytes. The method precision and intermediate precision was assessed by the overall % RSD.

Specificity (Selectivity) study is carried out to prove the ability of an analytical method to assess unequivocally the analyte in the presence of components which may be expected to be present in sample. The diluent, placebo solution, formoterol fumarate dihydrate selectivity solution, tiotropium selectivity solution, fumaric acid selectivity solution, standard and sample solution were prepared as mentioned in the analytical method, injected and recorded the observations for both TIO and FF.
In Forced degradation study, the sample and placebo were exposed under relevant stress conditions such as temperature, oxidation, photolytic, humidity, acid hydrolysis and base hydrolysis. Samples of these stress conditions were analyzed as per the analytical method described. The experiment was performed to achieve 5-30% of degradation in at least one stress condition.

**Linearity & Range:** Linearity of an analytical procedure is its ability within a given range to find test results which are directly proportional to the analyte concentration in the sample solution. TIO and FF standards were prepared in a range of LOQ to 150% of the working standard concentration. Linearity graph of concentration Vs average peak area of analyte was plotted separately. The correlation co-efficient, slope and y intercept were evaluated. The accuracy 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 obtained by the method. The samples for accuracy were prepared as per spiking the TIO and FF standard solution in placebo at LOQ level, 50%, 100% and 150% concentration levels of standard in triplicate for 50, 100, 150% and six times for LOQ level of working concentration and analysed as per the described method.

For filter Study, the sample solution was prepared as described in analytical method. The solution was centrifuged at 4000 rpm for 10 minutes. Decanted supernatant solution was injected as centrifuged sample solution. From the remaining half portion of the solution, Filtered the solution through 0.45 µm nylon filter and filled the vials by discarding 0mL, 2mL and 5mL of solution. These solutions were injected as sample solution. The peak responses were recorded for both the analytes for all centrifuged and filtered solution in single sequence. Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in the analytical method parameters and provides an indication of its reliability. In this study, Parameters like change in detection wavelength, flow rate, column oven temperature, mobile phase organic composition (Acetonitrile) and mobile phase buffer pH were performed and peak responses were recorded for both the analytes. For solution stability, the standard and sample solutions for both FF and TIO were prepared and injected against freshly prepared standard solution on day-0, day-1, day-2 and day-3.

**Results & Discussion**

**System suitability & System precision**
System suitability is demonstrated by preparing duplicate standard solution of TIO and FF and injecting the same. System precision is demonstrated by injecting standard solution of TIO and FF in six replicate injections according to the analytical method described above. For system suitability the similarity factor for both standard solution 1 and standard solution 2 should be between 95.0% to 105.0% for both TIO and FF. For system precision the similarity factor for six replicate injections of standard solution 1 should be between 95.0% to 105.0% for both TIO and FF. The number of theoretical plates should not be less than 2000, tailing factor should not be more than 2.0 and capacity factor should be more than 1.0 for both FF and TIO peaks. (Table 1 and Table 2).

**LOD and LOQ determination**
Prepare a series of standard preparations of FF and TIO standard over a range starting from 1% to at least 50% of standard concentrations (Fig.1). A series of low concentrations ranges from 0.007 ppm to 0.365 ppm for TIO and 0.005 ppm to 0.243 ppm for FF has been prepared based on standard response and injected in triplicate injections. The calibration curves were prepared for Area Vs Concentration for TIO and FF is given below. From these calibration curves slope; intercept and correlation coefficient from the Microsoft excel along with the STEYX were determined and the LOD & LOQ were calculated as per below formula (Table 3) (Fig.2-Fig.3).

For TIO,

\[
\text{LOD} = 3.3 \times \text{STEYX} / \text{Slope} = 3.3 \times 0.00241 = 0.008 \text{ PPM}
\]

\[
\text{Reported Value in PPM} = \text{NA}
\]

\[
\text{LOQ} = 10 \times \text{STEYX} / \text{Slope} = 10 \times 0.00241 = 0.024 \text{ PPM}
\]

\[
\text{Reported Value in PPM} = 0.015
\]

For FF,

\[
\text{LOD} = 3.3 \times \text{STEYX} / \text{Slope} = 3.3 \times 0.00210 = 0.007 \text{ PPM}
\]

From the prediction Linearity study statistically calculated LOD and LOQ values are, LOD is 0.008 ppm and LOQ is 0.024 ppm and reported LOQ = 0.015 ppm i.e. 0.02%.

For FF,

\[
\text{LOD} = 3.3 \times \text{STEYX} / \text{Slope} = 3.3 \times 0.00210 = 0.007 \text{ PPM}
\]
Reported Value in PPM = NA
LOQ = 10 x STEYX /Slope
= 10 x 0.00210
= 0.021 PPM
Reported Value in PPM = 0.01

From the prediction Linearity study statistically calculated LOD and LOQ values are, LOD is 0.007ppm and LOQ is 0.021 ppm and reported LOQ = 0.01ppm i.e. 0.02%.

Method Precision & Intermediate precision (Ruggedness)
In method precision, as per the analytical method six sample preparations were prepared representing a single batch. The intermediate precision or ruggedness was verified by performing precision study as per the analytical method six sample preparations of a single batch sample by different analyst, on different day, using different column and on different instrument. As per ICH guideline Q2 (R1), The % single maximum impurity (above LOQ Level), % total impurity, mean of % Single maximum impurity (above LOQ Level) and mean % total impurity for all twelve samples six of each method and intermediate precision were calculated the % RSD of results of % Single maximum impurity (above LOQ Level) & % total impurity of six sample preparations should not be more than 15.0 (Table 4).

Specificity (Selectivity)
Prepared diluent, placebo solution, FF Selectivity Solution, TIO Selectivity solution, Fumaric acid selectivity solution standard and sample solution as mentioned in analytical method and injected and recorded the observations. The diluent and placebo should not give any interfering peak at the retention time of FF and TIO peaks. The peak purity should pass for the both analyte peaks in standard and sample solution. Formoterol fumarate is a fumarate salt prepared from arformoterol, in a chemical reaction for every two molecules of formoterol one molecule of fumaric acid is released. Aim to inject Fumaric acid selectivity solution is to identify the retention time of fumaric acid and to confirm that it is not interfering with the retention time of FF and TIO peaks and based on the above observations the method is found to be selective (Table 5) (fig.4a - fig.4f).

Forced degradation
Forced degradation study is carried out to generate the data for the estimation of finished drug product stability. The forced degradation study consists of an appropriate solid and solution state stress conditions as per ICH guidelines. Intact capsules were kept at different stress conditions and were withdrawn at exact time and samples were prepared according to each conditions mentioned. The entire runtime was about double the retention times of both FF and TIO peaks. The degradant peaks should be well separated from the FF and TIO peaks also peak purity should pass for the both FF and TIO peaks in all the degradation samples as shown in (fig.5a - fig.5h). The sample and placebo were degraded in the following manner mentioned in (Table 6).

Linearity & range
The Linearity of related substance analytical method for FF and TIO in Formoterol fumarate and Tiotropium dry powder inhaler was performed in standard concentrations over the concentration levels ranging from LOQ to 150% of the standard solution standard concentration for each TIO and FF is considered as 100% that is 0.015 ppm to 1.089 ppm for TIO and 0.01ppm to 0.728ppm for FF. Linearity graph of concentration Vs average peak area of analytes plotted. The correlation coefficient between concentration (ppm), peak area slope and y intercept evaluated. Correlation coefficient should not be less than 0.999 for both analytes (Table 7) (Fig.6-Fig.7).

Accuracy
FF and TIO standards were spiked in placebo at different concentration levels i.e. LOQ level, 50%, 100% and 150% of targeted concentration and analyzed as per method described that is 0.0148ppm to 1.1129ppm for TIO and 0.01ppm to 0.7464ppm for FF. % Recovery obtained at concentration levels LOQ, 50%, 100% and 150% is reported in (Table 8).

At LOQ Level % recovery should be between 80.0 to 120.0% and % RSD of recovery at LOQ level should not more than 15.0 and at 50%, 100% and 150% level, % recovery should be between 85.0 to 115.0% and % RSD of recovery should not more than 15.0. The result observed are within the acceptance criteria, therefore the method is accurate throughout the selected range.

Filter Study
Prepared sample solution and analysed centrifuged and filtered sample solution through nylon filter 0.45µm in single sequence. The absolute % difference for % Single maximum impurity (above LOQ Level) and % total impurity between filtered and centrifuged sample solution should not be more than 2.0. Hence 0.45µm nylon
membrane filters can be used, and it is recommended to discard first 5 mL of the sample solution in the routine analysis (Table 9).

**Robustness**

The % RSD of the area of five replicate standard injections, theoretical plates and tailing factor of TIO peak in each replicate injection were recorded and reported (Table 10).

**Solution Stability**

The standard and sample solutions for FF and TIO were prepared on day 0 of experiment, stored these solutions at room temperature for every time interval up to 3 days and analyzed these solutions on subsequent days. The standard solution was prepared freshly and calculated the assay of analyte in the standard solution and % impurities in the sample solution.

Cumulative % RSD of % assay of the stored standard solution should not be more than 5.0. The % Single maximum impurity (above LOQ Level) & % total impurity for samples should comply with the specification limits. Cumulative % RSD of impurity results (above LOQ Level) obtained with stored sample solutions should not be more than 5.0.

The solution is considered stable, till the time point where the % RSD of the stored standard and sample Solution is not more than 5.0; Thus, the solution is stable up to 2 days at room temperature is proved (Table 11).

**Conclusion**

The recommended analytical method for the related substances determination of Tiomate transcaps® dry powder inhaler is simple, robust, selective, specific and precise. It also demonstrates the study of degradation pattern; therefore can be utilized for the quality control testing, routine analysis and for stability studies.

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Fig 1 – Overlaid Chromatogram of TIO & FF for LOD & LOQ determination 1% to 50%

Fig 2 - LOD & LOQ determination of FF

\[ y = 137.743,1890x + 83,6299 \]

\[ R^2 = 1.000 \]
Fig. 3 - LOD & LOQ determination of TIO

Tiotropium

\[ y = 227.482.0383x + 361.2807 \]

\[ R^2 = 1.000 \]

Fig. 4a – Chromatogram of (Specificity) Diluent

| No | Ret. Time (min) | Peak Name | Area (μAU*sec) | Peak Type | RRT | Height (μAU) | Area % | Match |
|----|----------------|-----------|----------------|-----------|-----|--------------|--------|-------|
| 1  | 1.525          | BMIS      | 2232           | n.a.      | 175 | 100.000      | 730    |
| Total |                |           | 2232           |           |     |              |        |       |
### Fig. 4b – Chromatogram of (Specificity) placebo solution

| No | Ret. Time (min) | Peak Name | Area (μAU*sec) | Peak Type | RRT | Height (μAU) | Area % | Match |
|----|----------------|-----------|----------------|-----------|-----|--------------|--------|-------|
| 1  | 11.544         |           | 1532           | BMS       | n.s.| 138          | 100.000| 625   |
|    | Total          |           | 1532           |           |     |              | 100    |       |

### Fig. 4c – Chromatogram of (Specificity) Tiotropium selectivity solution

| No | Ret. Time (min) | Peak Name  | Area (μAU*sec) | Peak Type | RRT | Height (μAU) | Area % | Match |
|----|----------------|------------|----------------|-----------|-----|--------------|--------|-------|
| 1  | 10.443         | Tiotropium | 10214683       | BMS       | 1.000 | 744830       | 100.000| 100   |
|    | Total          |            | 10214683       |           |     |              | 100    |       |
Fig. 4d – Chromatogram of (Specificity) Formoterol fumarate selectivity solution

| No | Ret. Time | Peak Name     | Area  | Peak Type | RRT | Height | Area % | Match |
|----|-----------|---------------|-------|-----------|-----|--------|--------|-------|
| 1  | 7.883     | Formoterol Fumarate | 5864146 | BMB | n.a. | 322421 | 99.855 | 1000  |
| 2  | 30.027    |               | 8460  | BMB | n.a. | 369   | 0.145  | 520   |
|    |           |               | Total |       |      | 5872837 |        | 100   |

Fig. 4e – Chromatogram of (Specificity) Fumaric acid selectivity solution

| No | Ret. Time | Peak Name | Area  | Peak Type | RRT | Height | Area % | Match |
|----|-----------|-----------|-------|-----------|-----|--------|--------|-------|
| 1  | 2.549     | Fumaric Acid | 632702 | BMB | n.a. | 79544 | 85.026 | 1000  |
| 2  | 3.621     |           | 87794 | BMB | n.a. | 1629  | 12.033 | 999   |
| 3  | 5.208     |           | 4370  | BMB | n.a. | 415   | 0.600  | 634   |
| 4  | 11.555    |           | 3204  | BMB | n.a. | 195   | 0.440  | 650   |
|    |           | Total     | 727861 |    |      |        |        | 100   |
Fig. 4f – Chromatogram of (Specificity) Sample solution

| No | Ret. Time (min) | Peak Name             | Area (µAU*sec) | Peak Type | RRT | Height (µAU) | Area % | Match |
|----|----------------|-----------------------|----------------|-----------|-----|--------------|--------|-------|
| 1  | 7.837          | Formoterol Fumarate   | 6240626        | BMB       | 0.752| 333107       | 29.762 | 009   |
| 2  | 10.419         | Tiotropium            | 15407360       | BMB       | 1.000| 794293       | 71.236 | 100   |
|    |                | Total                 | 21698086       |           |     |              | 100    |       |

Fig. 5a – Typical chromatogram of Diluent

| No | Ret. Time (min) | Peak Name | Area (µAU*sec) | Peak Type | RRT | Height (µAU) | Area % | Match |
|----|----------------|-----------|----------------|-----------|-----|--------------|--------|-------|
|    |                | Total     | 0              |           |     |              | 0      |       |
Fig 5b – Typical chromatogram of Standard Solution

| No | Peak Name          | Ret.Time | Area  | Peak Type | RRT | Area % | S/N Ratio |
|----|--------------------|----------|-------|-----------|-----|--------|-----------|
| 1  | Formoterol Fumarate| 7.480    | 66680 | BMB       | 0.735 | 28.736 | n.a.      |
| 2  | Tiotropium         | 10.182   | 165363| BMB       | 1.000 | 71.264 | n.a.      |
|    | Total              |          | 232043|           |      |        | 100       |

| No | Peak Name          | Ret.Time | Height | Resolution | Theoretical Plates | Asymmetry |
|----|--------------------|----------|--------|------------|---------------------|-----------|
| 1  | Formoterol Fumarate| 7.480    | 2519   | n.a.       | 3905                | 1.0       |
| 2  | Tiotropium         | 10.182   | 10631  | 6.2        | 10526               | 1.1       |
Fig 5c – Chromatogram of Photolytic degraded sample solution

| No | Ret. Time (min) | Peak Name       | Area (μAUσsec) | Peak Type | Height (μAU) | Area % | Match |
|----|----------------|-----------------|----------------|-----------|--------------|--------|-------|
| 1  | 7.275          |                  | 581            | BMB       | 0.700        | 375    | 0.018 | 940  |
| 2  | 7.821          | Formoterol Fumarate | 8121462        | BMB       | 0.753        | 330534 | 27.975 | 999  |
| 3  | 0.393          |                  | 7262           | BMB       | 0.807        | 570    | 0.033 | 918  |
| 4  | 10.362         | Tiotropium       | 10713808       | BMB       | 1.000        | 777248 | 71.812 | 1000 |
| 5  | 12.621         |                  | 8958           | BMB       | 1.215        | 886    | 0.055 | 941  |
| 6  | 37.688         |                  | 3210           | BMB       | 3.627        | 994    | 0.013 | 899  |
| 7  | 41.941         |                  | 10858          | BMB       | 4.036        | 1275   | 0.050 | 945  |
| 8  | 45.757         |                  | 9722           | BMB       | 4.493        | 920    | 0.044 | 839  |
| Total |           |                  | 21081808       |           |              | 100    |       |      |
Fig 5d – Chromatogram of Thermal degraded sample solution

| No | Ret. Time | Peak Name      | Area (µAU/sec) | Peak Type | RRT | Height (µAU) | Area % | Maltotriose |
|----|-----------|----------------|----------------|-----------|-----|--------------|--------|-------------|
| 1  | 7.251     | 25342          | 0.095          | 2049      | 0.122 | 999          |        |             |
| 2  | 7.832     | Formoterol Fumarate | 5875643      | BMB       | 0.759 | 358268      | 27.134 | 999         |
| 3  | 9.405     | 3767           | 0.905          | 774       | 0.043 | 929          |        |             |
| 4  | 10.395    | Tioctromium    | 1568552        | BMB       | 1.000 | 781436      | 72.485 | 1000        |
| 5  | 12.637    | 1343           | 1.216          | 1000      | 0.062 | 954          |        |             |
| 6  | 36.139    | 567            | 3.534          | 452       | 0.016 | 805          |        |             |
| 7  | 37.085    | 2220           | 3.625          | 463       | 0.015 | 802          |        |             |
| 8  | 45.733    | 2433           | 4.400          | 2152      | 0.112 | 981          |        |             |
| Total |     | 2165436        |                |          |      |              |        |             |
Fig 5c – Chromatogram of Humidity degraded sample solution

| No | Ret. Time (min) | Peak Name       | Area (µAU*sec) | Peak Type | RRT  | Height (µAU) | Area % | Match |
|----|----------------|-----------------|----------------|-----------|------|--------------|--------|-------|
| 1  | 7.781          | Formoterol Fumarate | 5737688        | BA3       | 0.953 | 352290       | 27.170 | 999   |
| 2  | 9.333          |                 | 5407           | BM2       | 0.504 | 703          | 0.400  | 874   |
| 3  | 10.328         | Tiotropium      | 10355160       | BM2       | 1.000 | 776204       | 72.713 | 1000  |
| 4  | 12.547         |                 | 12809          | BM2       | 1.215 | 963          | 0.010  | 914   |
| 5  | 32.005         |                 | 3894           | BM2       | 3.089 | 281          | 0.017  | 659   |
| Total |               |                 | 23147409       |           |      |              |        |       |
### Fig 5f – Chromatogram of Acid degraded sample solution

| No | Ret. Time (min) | Peak Name          | Area (μA.sec) | Peak Type | HRT (min) | Height (μA) | Area %  | Match |
|----|-----------------|--------------------|---------------|-----------|-----------|------------|----------|-------|
| 1  | 7.269           | Formoterol Fumarate| 27152         | BMB       | 0.709     | 2129       | 0.127    | 998   |
| 2  | 7.837           |                     | 5648700       | BMB       | 0.754     | 352090     | 27.334   | 999   |
| 3  | 9.416           |                     | 9554          | BMB       | 0.506     | 744        | 0.045    | 843   |
| 4  | 10.392          | Tiotropium          | 11466666      | BMB       | 1.008     | 775429     | 72.279   | 1000  |
| 5  | 12.619          |                     | 12697         | BMB       | 1.214     | 1021       | 0.051    | 954   |
| 6  | 16.525          |                     | 4217          | BMB       | 1.629     | 344        | 0.020    | 775   |
| 7  | 32.027          |                     | 190           | BMB       | 3.083     | 318        | 0.016    | 684   |
| 8  | 36.752          |                     | 6370          | BMB       | 3.337     | 594        | 0.030    | 751   |
| 9  | 45.741          |                     | 16004         | BMB       | 4.402     | 1876       | 0.080    | 977   |
|    | **Total**       |                    | **2139215**   |           |           |            | **100**  |       |
### Fig 5g – Chromatogram of Base degraded sample solution

![Chromatogram Image]

| No | Ret. Time (min) | Peak Name          | Area (μA*sec) | Peak Type | RRT | Height (μA) | Area % | Match |
|----|----------------|--------------------|---------------|-----------|-----|-------------|--------|-------|
| 1  | 7.821          | Formoterol Fumarate| 5900413       | BMB       | 0.753 | 252475      | 27.53% | 999   |
| 2  | 9.397          |                    | 6766          | BMB       | 0.995 | 3635        | 0.032  | 901   |
| 3  | 10.381         | Tildacrom           | 15504439      | BMB       | 1.990 | 768777      | 72.355 | 1000  |
| 4  | 12.024         |                    | 13516         | BMB       | 1.216 | 981         | 0.003  | 945   |
| 5  | 31.984         |                    | 4167          | BMB       | 3.081 | 325         | 0.019  | 747   |

Total: 25033082 μA*sec 100%
Fig 5h – Chromatogram of Hydrogen peroxide degraded sample solution

| No | Ret. Time (min) | Peak Name       | Area (μAU*sec) | Peak Type | RST | Height (μAU) | Area % | Match |
|----|----------------|-----------------|----------------|-----------|-----|--------------|--------|-------|
| 1  | 5.467          | Formoterol Fumarate | 18480         | BM9       | 0.526 | 1139         | 0.080  | 996   |
| 2  | 7.037          | Formoterol Fumarate | 752781        | BM9       | 0.754 | 270708       | 28.730 | 900   |
| 3  | 9.411          | BM9             | 7501          | BM9       | 0.929 | 898          | 0.096  | 803   |
| 4  | 10.019         | BM9             | 4728          | BM9       | 0.954 | 469          | 0.023  | 810   |
| 5  | 10.359         | BM9             | 11808914      | BM9       | 1.000 | 750783       | 72.984 | 1000  |
| 6  | 12.632         | BM9             | 26505         | BM9       | 1.234 | 1478         | 0.138  | 872   |
| 7  | 45.747         | BM9             | 6163          | BM9       | 4.401 | 572          | 0.030  | 822   |
| Total |                |                 | 2093091       |           |      |              |        | 100   |
Formoterol fumarate
\[ y = 139.302,5901x - 122,4944 \]
\[ R^2 = 0.9992 \]

Tiotropium
\[ y = 224.043,3645x + 2.446,2293 \]
\[ R^2 = 0.9994 \]

Table 1: System suitability & System Precision

| Injection          | Average area of 6 replicate standard injections | Standard deviation | % RSD |
|--------------------|------------------------------------------------|--------------------|-------|
| Standard solution -1 | 140777 | 1.1 | 10513 | 1615.5497 |
| Standard solution - 2 | 141361 | NA | NA | 1.15 |
Table 2: Linearity data for LOD & LOQ Determination

| LOD & LOQ Determination | Precision at LOQ Level |
|-------------------------|------------------------|
| Conc. In ppm            | Average area | Preparation | % Impurity |
| 0.007                   | 1657        | 1            | 0.0152     |
| 0.015                   | 3154        | 2            | 0.0157     |
| 0.037                   | 7936        | 3            | 0.0166     |
| 0.073                   | 16690       | 4            | 0.0160     |
| 0.147                   | 32844       | 5            | 0.0170     |
| 0.220                   | 48990       | 6            | 0.0175     |
| 0.367                   | 80005       | Average      | 0.0160     |
| Slope                   | 218829.9054 | Standard deviation | 0.0009 |
| Intercept               | 252.7574    | % RSD        | 5.63       |
| Correlation Coefficient | 1000        |              |            |
| STEYX                   | 527.46      |              |            |
| STEYX/Slope             | 0.00241     |              |            |

Table 3: Method Precision, Intermediate Precision

| Preparation   | % Single Maximum Impurity | % Total Impurity |
|---------------|---------------------------|-----------------|
| Method Precision |                          |                 |
| 1             | 0.109                     | 0.207           |
| 2             | 0.122                     | 0.223           |
| 3             | 0.132                     | 0.267           |
| 4             | 0.129                     | 0.244           |
| 5             | 0.133                     | 0.255           |
| 6             | 0.133                     | 0.261           |
| **Average (A)** | 0.128             | **0.243**     |
| Standard deviation | 0.0116               | 0.0234          |
| % RSD          | **9.06**                 | **9.63**       |
| Intermediate Precision |                  |                 |
| 7             | 0.101                     | 0.194           |
| 8             | 0.123                     | 0.239           |
| 9             | 0.131                     | 0.258           |
| 10            | 0.121                     | 0.233           |
| 11            | 0.134                     | 0.257           |
| 12            | 0.126                     | 0.245           |
| **Average (B)** | 0.123             | **0.238**     |
| Standard deviation | 0.0117               | 0.0235          |
| % RSD          | **9.51**                 | **9.87**       |
| Overall Average (A+B) | 0.126             | 0.240           |
| Overall Standard deviation | 0.0115 | 0.0225       |
| % RSD          | **9.13**                 | **9.38**       |
### Table 4: Selectivity

| Sr. No. | Solution Preparation       | Observation at Retention time of Product                                                                 | Peak Purity match (TIO) | Peak Purity match (FF) | Peak Purity Results |
|--------|-----------------------------|----------------------------------------------------------------------------------------------------------|-------------------------|------------------------|---------------------|
| 1      | Diluent                     | No Interference is observed at the retention time of Formoterol and Tiotropium peaks.                    | NA                      |                        |                     |
| 2      | Placebo solution            | No Interference is observed at the retention time of Formoterol and Tiotropium peaks                   | NA                      |                        |                     |
| 3      | Formoterol fumarate dihydrate Selectivity Solution | Peak purity passes & no interference observed at the retention time of Tiotropium peak and impurity peaks. | 1000                    | 1000                   | Passes              |
| 4      | Tiotropium Selectivity Solution | Peak purity passes & no interference observed at the retention time of Formoterol Fumarate peak and impurity peaks. | 1000                    | 1000                   | Passes              |
| 5      | Fumaric acid selectivity solution | Peak purity passes & no interference observed at the retention time of Formoterol Fumarate and Tiotropium peak and impurity peaks. | 1000                    | 1000                   | Passes              |
| 6      | Standard Solution           | Peak purity of Formoterol and Tiotropium peaks passes.                                                 | 999                     | NA                     | Passes              |
| 7      | Sample Solution             | Peak purity of Formoterol and Tiotropium peaks passes.                                                 | 1000                    | 999                    | Passes              |

### Table 5: Forced degradation

| Sr. No. | Degradation Condition | Degradation agents / condition | Exposure period | % Single Maximum Impurity | % Total degraded Impurities | Peak Purity match (TIO) | Peak Purity match (FF) | Peak purity Result |
|---------|-----------------------|--------------------------------|-----------------|---------------------------|-----------------------------|-------------------------|-----------------------|---------------------|
| 1       | Thermal               | 60°C for 2 Days, 1.2 million lux hours; 200 watt hrs./m² | For 2 Days      | 0.189                     | 0.575                        | 1000                    | 999                   | Passes              |
| 2       | Photolytic            | 1.2 million lux hours; 200 watt hrs./m² | For 7 days      | 0.086                     | 0.336                        | 1000                    | 999                   | Passes              |
| 3       | Humidity              | 40°C/75% RH for 7 days         | For 7 days      | 0.092                     | 0.179                        | 1000                    | 999                   | Passes              |
| 4       | Acid                  | 0.01N HCl for 1 Hr. at RT     | For 1 Hr. at RT | 0.196                     | 0.597                        | 1000                    | 999                   | Passes              |
| 5       | Base                  | 0.001N NaOH for 5 min. at RT  | For 5 min. at RT | 0.098                     | 0.177                        | 1000                    | 999                   | Passes              |
| 6       | Peroxide              | 3% H2O2 for 24 Hr. at RT      | For 24 Hr. at RT | 0.206                     | 0.458                        | 1000                    | 999                   | Passes              |
Table 6: Linearity

| Linearity Level | Conc. (%) | Conc. (ppm) | Area     |
|-----------------|-----------|-------------|----------|
| 1               | LOQ       | 0.015       | 3344     |
| 2               | 20        | 0.145       | 33164    |
| 3               | 50        | 0.363       | 82961    |
| 4               | 80        | 0.581       | 132931   |
| 5               | 100       | 0.726       | 167583   |
| 6               | 120       | 0.872       | 199501   |
| 7               | 150       | 1.089       | 250118   |

Slope: 229742.0847
Intercept: -192.8919
Correlation Coefficient: 1.000

Graph 1: Linearity graph of TIO

![Linearity Graph](image)

Table 7: Accuracy

| Preparation | Amount added (ppm) | Amount Recovered (ppm) | % Recovery |
|-------------|--------------------|------------------------|------------|
| 1           | 0.0150             | 0.0142                 | 94.7       |
| 2           | 0.0150             | 0.0146                 | 97.3       |
| 3           | 0.0150             | 0.0155                 | 103.3      |
| 4           | 0.0150             | 0.0149                 | 99.3       |
| 5           | 0.0150             | 0.0158                 | 105.3      |
| 6           | 0.0150             | 0.0163                 | 108.7      |
| Average     | 0.0150             | 0.0163                 | **101.4**  |
Table 8: Filter validation

| Sample Solution | % Impurity | Absolute % Difference |
|-----------------|------------|-----------------------|
|                 | % Single maximum impurity | % Total Impurity | % Single maximum impurity | % Total Impurity |
| Centrifuged     | 0.105      | NA                    | NA                      |
| 0 mL discarded  | 0.106      | 0.347                 | 0.95                    | 70.65              |
| 2 mL discarded  | 0.106      | 0.202                 | 0.95                    | 0.50               |
| 5 mL discarded  | 0.105      | 0.201                 | 0.00                    | 0.00               |

Table 9: Robustness

| Parameters | Wavelength (nm) (+/-3) | Flow rate (mL/min) (+/-0.1mL/min) | Column Temperature (°C) (+/- 5°C) | Gradient composition (+/- 5%) | Buffer pH (+/- 0.2) |
|------------|------------------------|-----------------------------------|-----------------------------------|--------------------------------|---------------------|
|            | 23°C                   | 24°C                              | 0.9                               | 1.1                            | 25°C                |
| Similarity factor | 98.5                  | 96.9                              | 100.1                             | 100.7                          | 99.0                |
| T.F.       | 1.2                    | 1.2                               | 1.2                               | 1.2                            | 1.3                 |
| NTP        | 10063                  | 9990                              | 10951                             | 8797                           | 10828               | 9817                | 10952               | 10082               | 9638                | 9110                |
| Time point | Stability data for Standard solution | Stability data for Sample solution |
|------------|------------------------------------|----------------------------------|
|            | % TIO | Cumulative | % Single Maximum Impurity | Cumulative | % Total Impurity | Cumulative |
|            | Avg.  | SD         | %RSD | Avg.  | SD | %RSD | Avg.  | SD | %RSD |
| Day 0 (Initial) | 100.0 | NA | NA | NA | 0.109 | NA | NA | NA | 0.207 | NA | NA | NA |
| Day 1      | 101.5 | 100.8 | 1.0607 | 1.05 | 0.104 | 0.107 | 0.0035 | 3.27 | 0.205 | 0.206 | 0.0014 | 0.68 |
| Day 2      | 96.1  | 99.2 | 2.7875 | 2.81 | 0.102 | 0.105 | 0.0036 | 3.43 | 0.191 | 0.201 | 0.0087 | 4.33 |