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ORIGINAL ARTICLE

Quantitative Determination of Related Substances in Formoterol Fumarate and Tiotropium in Tiomate Transcaps® Dry Powder Inhaler

Tiomate Transcaps® Kuru Toz İnhalerde Formoterol Fumarat ve Tiotropiumdaki İlgili Maddelerin Kantitatif Tayini

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ABSTRACT

Objectives: Tiotropium (TIO) and formoterol fumarate (FF) combination in dry powder inhaler (DPI) dosage form used for treating asthma, bronchospasm, chronic bronchitis, emphysema and chronic obstructive pulmonary diseases. Aim to develop an analytical method for estimating emerging and advancing dry powder inhaler combination toward 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 reverse phase-high performance liquid chromatography (HPLC) method has been developed for the determination of related substances in FF and TIO DPI.

Materials and Methods: The analytical method was performed on schimadzu HPLC with a quaternary pump, the separation achieved using BDS Hypersil C18 (250×4.6 mm, 5 µm) column with mobile phase consisting of sodium phosphate buffer pH 3.2 and acetonitrile 1.0 mL min^{-1} flow rate in the gradient elution. Diluent consists of a mixture of buffer pH 3.2 and acetonitrile in the ratio of (70:30) %v/v. 30˚C column temperature and photodiode-array detection detector at a wavelength 240 nm. The run time was 50 min. The Retention time of FF and TIO was found to be at 7.8 and 10.3 min respectively.

Results: Both the analyte peaks were found to be free from interference. The method was validated as *per* the International Council on Harmonisation guidelines, the linearity was performed on 0.015 to 1.089 ppm for TIO and 0.01 to 0.728 ppm for FF concentration with correlation coefficient of 1,000. The precision and accuracy were also performed at the limit of quantification level were within the limits. Forced degradation study was also conducted.

Conclusion: The recommended method for the related substance determination of FF and TIO is simple, selective, specific and precise. It also demonstrates the study of the degradation pattern. Moreover, the above developed related substance analytical method was applied to the bulk analysis and pharmaceutical dosage form for routine analysis and stability study.

Key words: Dry powder inhaler, forced degradation study, LOD & LOQ determination, ICH guidelines, asthma, COPD (chronic obstructive pulmonary diseases)

ÖZ

Amaç: Kuru toz inhaler (KTİ) dozaj formunda olan tiotropium (TIO) ve formoterol fumarat (FF) kombinasyonu, astım, bronkospazm, kronik bronşit, amfizem ve kronik obstrüktif akciğer hastalıklarının tedavisinde kullanılmaktadır. Amaç, kuru toz inhaler kombinasyonları olarak ortaya çıkan ve gelişmekte olan dozaj formlarındaki ilgili maddelerin tespitine yönelik analitik bir yöntem geliştirmektir. KTİ'de bulunan FF, TIO ve ilgili maddelerin belirlenebilmesi için öncelikle hassas, basit, sağlam ve valide edilmiş yeni bir ters faz yüksek performanslı sıvı kromatografisi (HPLC) yöntemi geliştirilmiştir.

Gereç ve Yöntemler: Analitik yöntem, dörtlü pompalı Shimadzu HPLC cihazı ile gerçekleştirilmiştir; ayırım BDS Hypersil C18 (250x4,6 mm, 5 µm) kolonu, 1,0 mL dk^{-1} akış hızında sodyum fosfat tamponu pH 3,2 ve asetonitrilden oluşan mobil faz kullanılarak gradient elüsyon ile sağlanmıştır. Mobil faz pH 3,2 tampon ve asetonitril karışımından (70:30; % h/h) oluşmaktadır. Analiz, kolon sıcaklığı 30˚C, fotodiyot dizi detektörü 240 nm dalga boyunda gerçekleştirilmiştir. Analiz süresi 50 dk olmuştur. FF ve TIO'nun retansiyon zamanları sırasıyla 7,8 ve 10,3 dk olarak bulunmuştur.

Bulgular: Her iki analitin herhangi bir girişimde bulunmadığı saptanmıştır. Yöntem, Uluslararası Harmonizasyon Konseyi yönergelerine göre valide edilmiştir; doğrusallık TIO için 0,015-1,089 ppm ve FF için 0,01-0,728 ppm konsantrasyon aralıklarında, 1,000 korelasyon katsayısı ile

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gerçekleştirilmiştir. Kesinlik ve doğruluk, tayin limiti seviyesinde gerçekleştirilmiş, sınırlar içinde bulunmuştur. Zorlamalı bozunma çalışması da gerçekleştirilmiştir.

Sonuç: FF, TIO ve ilgili maddelerin tespiti için önerilen yöntem basit, seçici, spesifik ve kesindir. Aynı zamanda zorlamalı degradasyon çalışmasını da göstermektedir. Ayrıca, geliştirilen analitik yöntem yığın analizi ve farmasötik dozaj formunun rutin analizlerinde ve stabilite çalışmasına uygulanmıştır.

Anahtar kelimeler: Kuru toz inhaler, zorlamalı degradasyon çalışması, LOD & LOQ belirleme, ICH kılavuzu, astım, KOAH (kronik obstrüktif akciğer hastalığı)

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 formoterol fumarate (FF) and tiotropium (TIO) is used in targeting various characteristics of COPD as bronchodilation and the inflammations.[1,2]

FF dihydrate is a directly acting sympathomimetic with beta-adrenoceptor stimulant activity. FF is prescribed for its long acting beta 2 agonist effect for treating airway obstruction, asthma and COPD.[3] The pharmacological effect of beta 2 agonist is to stimulate intracellular adenyl cyclase enzyme that catalyzes the conversion of adenosine triphosphate 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-(1*RS*)-1-hydroxy-2-(1*RS*)-2(4methoxyphenyl)1methy lethylaminoethyl phenyl formamide(*E*)-butenedioatedihydrate with molecular formula $C_{42}H_{52}N_4O_{12}\cdot 2H_2O$ and molecular weight of 840.92.[1,2]

TIO bromide monohydrate is an anticholinergic, antimuscarinic bronchodilator used in the airway obstruction, COPD conditions.[1-3] TIO shows its pharmacological effects by inhibiting M3 receptors in the smooth muscle, which leads to bronchodilation. Chemically it is (1*R*,2*R*,4*S*,5*S*,7*s*)-7-(2-hydroxy-2,2-dithiophen-2-ylacetyl)oxy-9,9-dimethyl-3-oxa-9-azoniatricyclo3.3.1.02,4 non-anebromidemonohydrate with molecular formula $C_{19}H_{22}BrNO_4S_2\cdot H_2O$ and molecular weight of 490.40.[1]

A complete literature survey reveals that TIO is determined by spectrophotometric method.[4] TIO in bulk and dry powder inhalation (DPI) form is determined by high performance thin-layer chromatography.[5] Methods are available to determine TIO and related substances by high performance liquid chromatography (HPLC).[6] For the biological estimation of TIO in human plasma; three methods illustrated.[7-9] Estimation of FF in various pharmaceutical dosage forms 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 technique.[13] FF also estimated along with other drug moieties by thin layer chromatography densitometry methods.[14-17] FF also estimated along with other drug moieties in HPLC,[14,17-24] also in plasma, urine and biological samples.[25,26] TIO has been determined by either FF[27-29] or ciclesonide or olodaterol[30-33] in various dosage forms by HPLC methods, but the focus was found to be on a single drug compound. In FF the hydrazine hydrate content is determined by gas chromatography-mass spectrometry method.[34] Moreover, no related substances analytical method available in any of the pharmacopeias.

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 DPI dosage form and validated according to International Council for Harmonisation (ICH) guidelines.[35] The proposed validated reversed-phase-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® DPI manufactured by Lupin ltd. India is used.

MATERIALS AND METHODS

Instrumentation

The Dionex HPLC system consists of dionex ultimate 3,000 UHPLC system equipped with a quaternary gradient pump dionex ultimate 3,000 pumps, dionex ultimate 3,000 auto sampler, dionex ultimate 3,000 column compartment and a dionex ultimate 3,000 UV-Photo Diode Array detector. Separation and quantitation were carried out using a C18 Hypersil BDS column (250 mmx4.6 mm, 5 µm) Chromeleon 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 utilizes a gradient elution in which buffer consists of 1.38 mg of sodium dihydrogen phosphate monohydrate in 1,000 mL of water, add 2 mL 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 30˚C column temperature and photodiode-array detection (PDA) detector at wavelength 240 nm 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.

The 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 36-mg TIO separately in each 100 mL volumetric flask, added 70 mL of diluent sonicate to dissolve and make volume with diluent and mix. Further dilute 5 mL of this solution to 100 mL with the diluent.

FF standard stock solution

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

Mix standard solution

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

Sample preparation

Tiomate Transcaps® (Lupin ltd..) preparation, carefully open and collect the sample powder equivalent to 0.72-mg TIO in to 10 mL volumetric flask, added about 7 mL 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 peak area due to diluent, FF and placebo solution in the sample solution. Calculate the % of each impurity present in the sample solution by following formulae:

Calculation:

$$\text{Similarity factor} = \frac{\text{Area of standard -1}}{\text{Area of standard -2}} \times \frac{\text{Wt. of standard -2}}{\text{Wt. of standard -1}} \times 100$$

$$\textbf{\% Impurity} = \frac{AT}{AS} \times \frac{\text{Wt. std}}{100} \times \frac{5}{100} \times \frac{5}{100} \times \frac{10}{\text{Wt. spl}} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{L.C.}} \times \frac{392.5}{490.4} \times 100 \times 1000$$

where,

AT: Area of each impurity in the sample solution, As: The following: 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 by using various C8 and C18 columns but no proper separations were achieved. Different proportions of potassium and sodium salt buffer (10 mMol to 30 mMol) with methanol and/or acetonitrile were used in various proportions in both isocratic and gradient elution patterns but no proper peak shape, tailing factor and theoretical plates of TIO and FF were 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 were 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 30˚C column temperature and PDA detector at a wavelength 240 nm 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, limit of detection (LOD) & limit of quantification (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 was 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 LOD & LOQ from linearity study were determined.

Method precision may be defined as the precision of an analytical procedure expressing the closeness of agreement between a series of measurements 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 % relative standard deviation (RSD).

Intermediate precision (ruggedness) expresses the ability of an analytical method to remain unaffected and produce reliable results within the laboratory variations such as different days, different equipment, different analysts. 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.

The specificity (selectivity) study is conducted 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 the sample. The diluent, placebo solution, FF dihydrate selectivity solution, TIO 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; the linearity of an analytical procedure is its ability within a given range to find test results that 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 the 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 using the method. The samples for accuracy were prepared as *per* spiking the TIO and FF standard solution in the placebo at LOQ level, 50%, 100%, and 150% concentration level 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 the filter study, the sample solution was prepared as described in the analytical method. The solution was centrifuged at 4,000 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 0 mL, 2 mL and 5 mL solution. These solutions were injected as a sample solution. The peak responses were recorded for both the analytes for all centrifuged and filtered solution in single sequence.

The 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 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 AND 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 2,000, 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, 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 (Figure 1). A series of low concentrations

Table 1. System suitability

		Area	Similarity factor	Tailing factor	Theoretical plates	Capacity factor
Formoterol fumarate	Standard solution-1	66680	100.0	1.0	3905	1.58
	Standard solution-2	66567		1.0	3973	1.57
Tiotropium	Standard solution-1	165363	100.1	1.1	10526	2.51
	Standard solution-2	165256		1.1	10647	2.51

Table 2. System precision

System precision FF					System precision TIO				
Inj. no	**FF area**	**Similarity factor**	**TF**	**NTP**	**Inj. no**	**TIO area**	**Similarity factor**	**TF**	**NTP**
1	66680	100.0	1.0	3905	1	165363	100.0	1.1	10526
2	66769	99.9	1.0	3969	2	165007	100.2	1.1	10569
3	66800	99.8	1.0	3980	3	165529	99.9	1.1	10553
4	66518	100.2	1.0	3969	4	165026	100.2	1.1	10641
5	66114	100.9	1.0	3971	5	165902	99.7	1.1	10533
6	66422	100.4	1.0	3990	6	166040	99.6	1.1	10656
Avg.	**66551**				**Avg.**	**165478**			
SD	258.7908				STDEV	433.0337			
% RSD	**0.39**				**% RSD**	**0.26**			

FF: Formoterol fumarate, TIO: Tiotropium, SD: Standard deviation, RSD: Relative standard deviation, TF: Tailing factor, NTP: Number of theoretical plates, Inj. no: Injection number, Avg: Average

ranges from 0.007 ppm to 0.365 ppm for TIO and 0.005 ppm to 0.243 ppm for FF has been prepared on the basis of 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 curve 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, Figure 2, 3).

For TIO,

LOD = 3.3 x STEYX/slope
=3.3 x 0.00241
=0.008 PPM

Reported value in PPM = NA

LOQ =10 x STEYX/slope
= 10 x 0.00241
= 0.024 PPM

Reported value in PPM = 0.015

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,

LOD =3.3 x STEYX/slope
=3.3 x 0.00210
=0.007 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 0.007 ppm and LOQ is 0.021 ppm and reported LOQ = 0.01 ppm *i.e.* 0.02%.

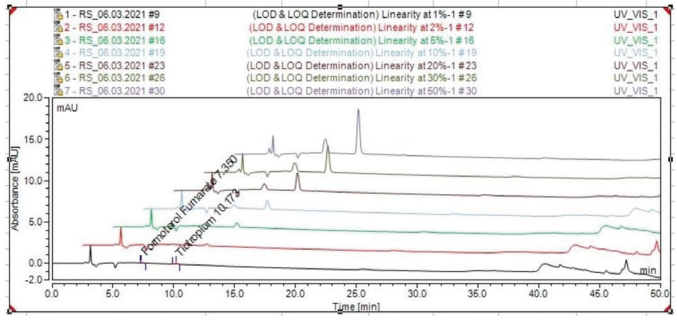


Figure 1. Overlaid chromatogram of TIO & FF for LOD & LOQ determination 1% to 50%

TIO: Tiotropium, FF: Formoterol fumarate, LOD: Limit of detection, LOQ: Limit of quantification

Method precision & intermediate precision (ruggedness)

In method precision, as *per* the analytical method, six sample preparation were prepared representing a single batch. The intermediate precision or ruggedness was verified by performing precision study as *per* the analytical method six sample preparation 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, the mean of % single maximum impurity (above LOQ level), 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

Table 3. Linearity data for LOD & LOQ determination

FF LOD & LOQ determination		FF precision at LOQ level	
Conc. in ppm	**Average area**	**Preparation**	**% Impurity**
0.005	707	1	0.0190
0.010	1366	2	0.0199
0.024	3366	3	0.0190
0.049	6586	4	0.0191
0.097	13923	5	0.0196
0.146	20410	6	0.0180
0.243	33292	**Average**	**0.0190**
Slope	**137743.1890**	Standard deviation	0.0007
Intercept	**83.6299**	**% RSD**	**3.68**
Correlation coefficient	**1.000**		
STEYX	**289.66**		
STEYX/slope	**0.00210**		
TIO LOD & LOQ determination		**TIO precision at LOQ level**	
Conc. in ppm	**Average area**	**Preparation**	**% Impurity**
0.007	1777	1	0.0160
0.015	3611	2	0.0166
0.036	8390	3	0.0164
0.073	16835	4	0.0161
0.146	34770	5	0.0157
0.219	49835	6	0.0158
0.365	83173	**Average**	**0.0160**
Slope	**227482.0383**	Standard deviation	0.0003
Intercept	**361.2807**	**% RSD**	**1.88**
Correlation coefficient	**1.000**		
STEYX	**582.95**		
STEYX/slope	**0.00256**		

LOD: Limit of detection, LOQ: Limit of quantification, FF: Formoterol fumarate, RSD: Relative standard deviation, STEYX: Standard error of estimates, TIO: Tiotropium, Conc.: Concentration

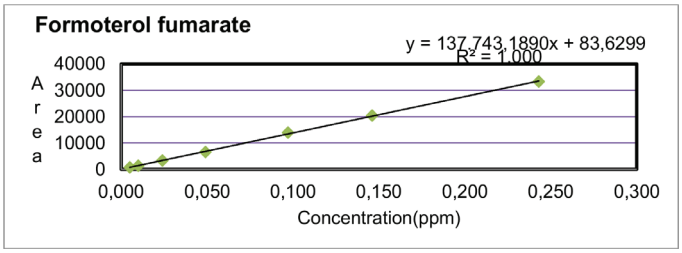


Figure 2. LOD & LOQ determination of FF

FF: Formoterol fumarate, LOD: Limit of detection, LOQ: Limit of quantification

FF and TIO peaks. The peak purity should pass for the analyte peaks in the standard and sample solution. FF 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, Figure 4a-f).

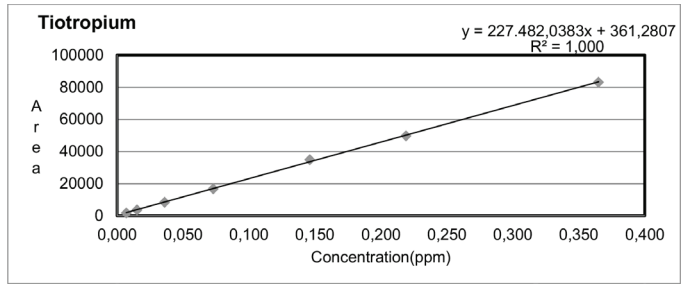


Figure 3. LOD & LOQ determination of TIO

TIO: Tiotropium, LOD: Limit of detection, LOQ: Limit of quantification

Table 4. Method precision, intermediate precision

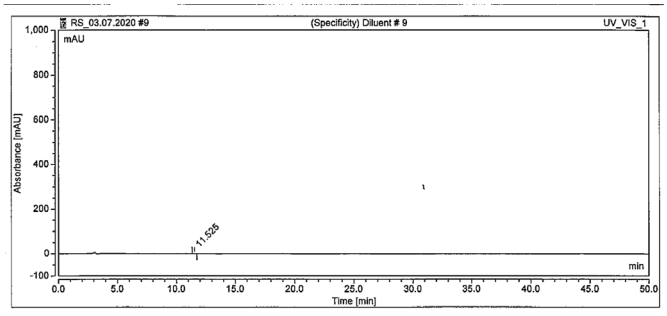
Preparation		% Single maximum impurity	% Total impurity
Method precision	1	0.109	0.207
	2	0.122	0.223
	3	0.142	0.267
	4	0.129	0.244
	5	0.135	0.255
	6	0.133	0.261
Average (A)		**0.128**	**0.243**
Standard deviation		0.0116	0.0234
% RSD		**9.06**	**9.63**
Intermediate precision	7	0.101	0.194
	8	0.123	0.239
	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**

RSD: Relative standard deviation

Table 5. Selectivity

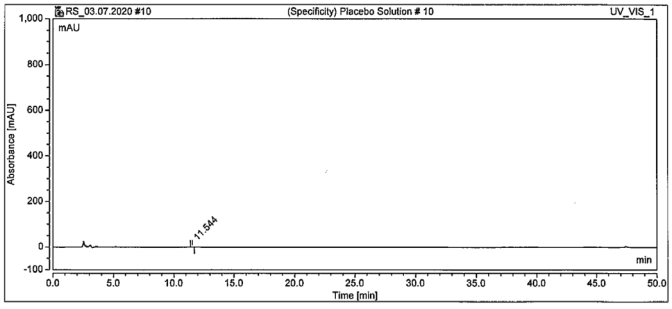
Sr. no.	Solution preparation	Observation at retention time of product	Peak purity match (TIO)	Peak purity match (FF)	Peak purity results
1	Diluent	No interference is observed at the retention time of formoterol and tiotropium peaks	NA		
2	Placebo solution	No interference is observed at the retention 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. % Single maximum impurity (above LOQ level): 0.093. % Total impurity: 0.167	1000	999	Passes

TIO: Tiotropium, LOQ: Limit of quantification, FF: Formoterol fumarate, Sr.: Serial number, NA: Not applicable



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	11.525		2232	BMB	n.a.	175	100.000	730
	Total		2232				100	

Figure 4a. Chromatogram of (specificity) diluent

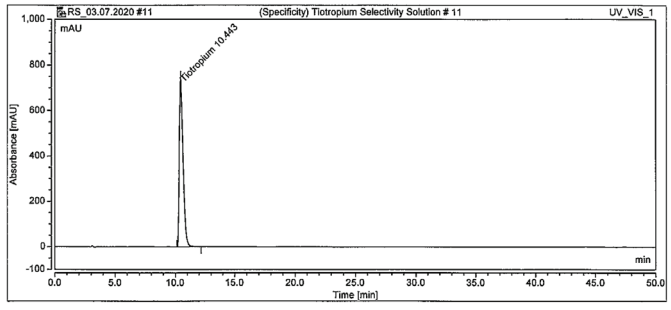


No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	11.544		1532	BMB	n.a.	138	100.000	625
	Total		1532				100	

Figure 4b. Chromatogram of (specificity) placebo solution

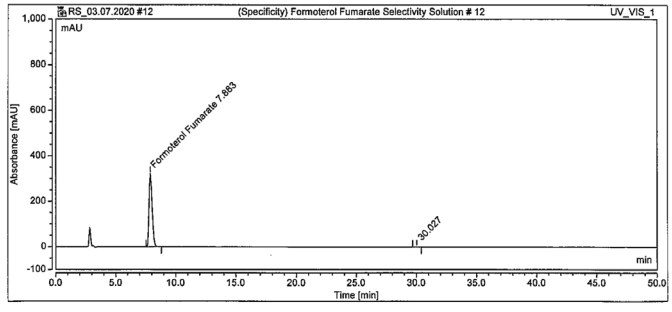
Forced degradation

Forced degradation study is conducted to generate the data for estimating finished drug product stability. The forced degradation study consists of an appropriate solid and solution state stress conditions as *per* ICH guidelines. Intact capsules



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	10.443	Tiotropium	16214683	BMB	1.000	744630	100.000	1000
	Total		16214683				100	

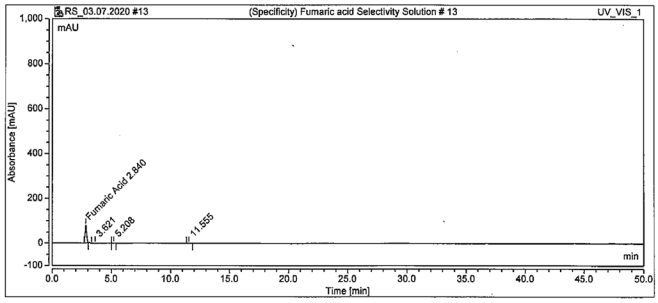
Figure 4c. Chromatogram of (specificity) tiotropium selectivity solution



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.883	Formoterol Fumarate	5864148	BMB	n.a.	322421	99.855	1000
2	30.027		8490	BMB	n.a.	389	0.145	800
	Total		5872637				100	

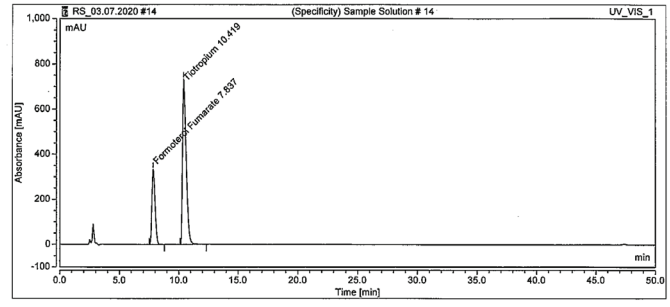
Figure 4d. Chromatogram of (specificity) formoterol fumarate selectivity solution

were kept at different stress conditions and were withdrawn at the exact time and samples were prepared according to each condition mentioned. The entire runtime was about double the retention times of both FF and TIO peaks. The degradant peaks



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	2.840	Fumaric Acid	632702	BMB	n.a.	79544	86.926	1000
2	3.621		87584	BMB	n.a.	1629	12.033	990
3	5.208		4370	BMB	n.a.	415	0.600	934
4	11.555		3204	BMB	n.a.	195	0.440	650
	Total		727861				100	

Figure 4e. Chromatogram of (specificity) fumaric acid selectivity solution



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.837	Formoterol Fumarate	6240826	BMB	0.752	333107	28.762	999
2	10.419	Tiotropium	15457260	BMB	1.000	734295	71.238	1000
	Total		21698086				100	

Figure 4f. Chromatogram of (specificity) sample solution

should be well separated from the FF and TIO peaks also peak purity should pass for the FF and TIO peaks in the degradation samples as shown in (Figure 5a-h). The sample and placebo were degraded in the following manner mentioned in (Table 6).

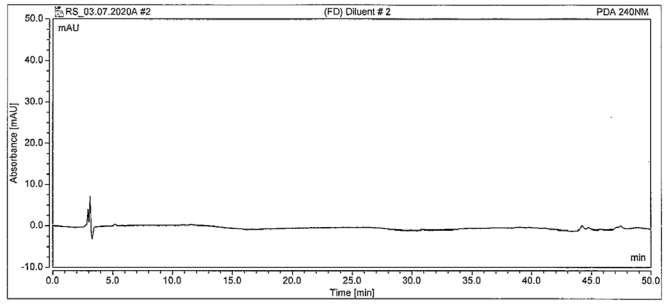
Linearity & range

The linearity of related substance analytical method for FF and TIO in FF and TIO DPI 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 100% that is 0.015 ppm to 1.089 ppm for TIO and 0.01 ppm to 0.728 ppm 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. The correlation coefficient should not be less than 0.999 for both analytes (Table 7, Figure 6, 7).

Accuracy

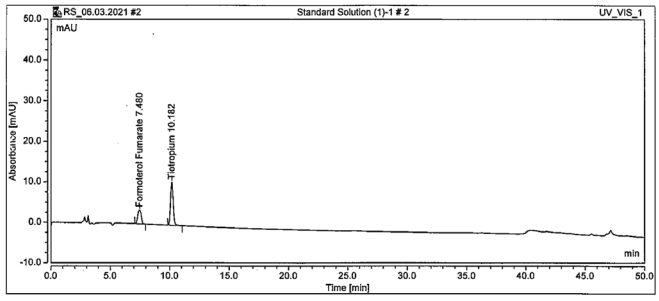
FF and TIO standards were spiked in placebo at different concentration levels *i.e.* LOQ level, 50%, 100% and 150% of targeted concentration and analyzed as *per* method described that is 0.0148 ppm to 1.1129 ppm for TIO and 0.01 ppm to 0.7464 ppm for FF. % recovery obtained at concentration levels LOQ,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



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
	Total		0				0	

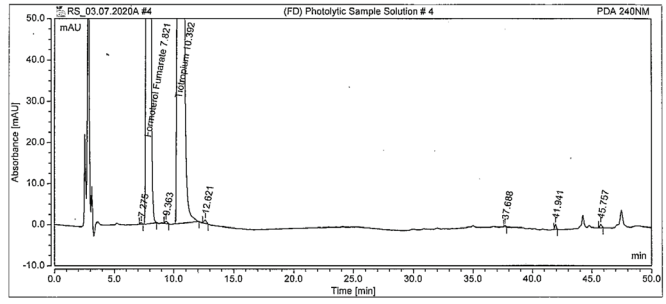
Figure 5a. Typical chromatogram of diluent



No	Peak Name	Ret.Time min	Area µAU*sec	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 min	Height µAU	Resolution (USP)	Theoretical Plates	Asymmetry
1	Formoterol Fumarate	7.480	3319	n.a.	3905	1.0
2	Tiotropium	10.182	10631	6.2	10526	1.1

Figure 5b. Typical chromatogram of standard solution



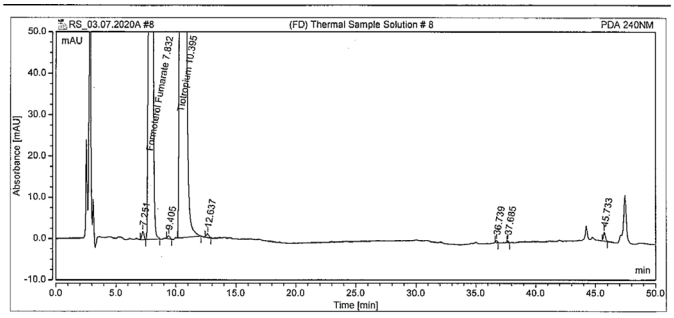
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.275		3981	BMB	0.700	375	0.018	940
2	7.821	Formoterol Fumarate	6121462	BMB	0.753	330534	27.975	999
3	9.363		7282	BMB	0.901	570	0.033	918
4	10.392	Tiotropium	15713808	BMB	1.000	777249	71.812	1000
5	12.621		11968	BMB	1.215	886	0.055	941
6	37.688		2826	BMB	3.627	394	0.013	809
7	41.941		10838	BMB	4.036	1275	0.050	895
8	45.757		9722	BMB	4.403	920	0.044	839
	Total		21881888				100	

Figure 5c. Chromatogram of photolytic degraded sample solution

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.

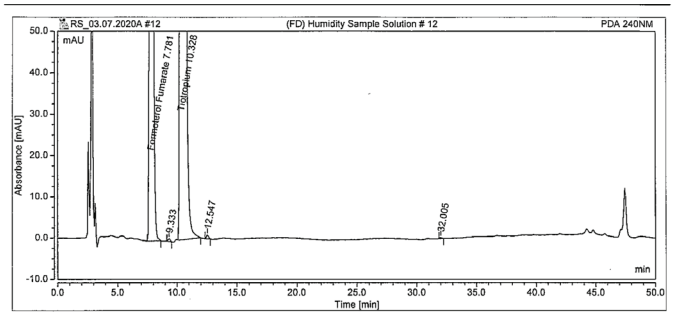
Filter study

The prepared sample solution and analysed centrifuged and filtered sample solution through nylon filter 0.45 µm in single



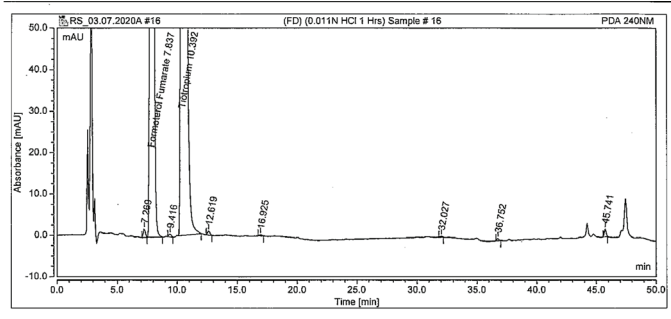
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.251		26342	BMB	0.698	2049	0.122	999
2	7.832	Formoterol Fumarate	5875643	BMB	0.753	358268	27.134	999
3	9.405		9267	BMB	0.905	774	0.043	929
4	10.395	Tiotropium	15698262	BMB	1.000	781496	72.495	1000
5	12.637		13434	BMB	1.216	1000	0.062	954
6	36.739		3837	BMB	3.534	452	0.018	805
7	37.685		3220	BMB	3.625	463	0.015	802
8	45.733		24339	BMB	4.400	2152	0.112	981
	Total		21654344				100	

Figure 5d. Chromatogram of thermal 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	BMB	0.753	352290	27.170	999
2	9.333		8407	BMB	0.904	703	0.040	876
3	10.328	Tiotropium	15355160	BMB	1.000	776204	72.713	1000
4	12.547		12603	BMB	1.215	963	0.060	910
5	32.005		3551	BMB	3.099	281	0.017	659
	Total		21117409				100	

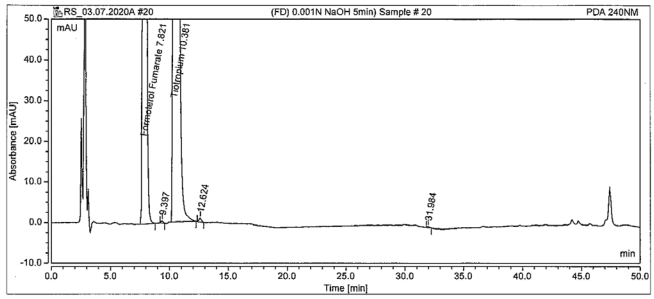
Figure 5e. Chromatogram of humidity degraded sample solution



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.269		27152	BMB	0.700	2129	0.127	998
2	7.837	Formoterol Fumarate	5848700	BMB	0.754	352096	27.334	999
3	9.416		9554	BMB	0.906	744	0.045	843
4	10.392	Tiotropium	15465666	BMB	1.000	775429	72.279	1000
5	12.619		12987	BMB	1.214	1021	0.061	954
6	16.925		4217	BMB	1.629	344	0.020	775
7	32.027		3490	BMB	3.082	318	0.016	684
8	36.752		6379	BMB	3.537	594	0.030	751
9	45.741		19004	BMB	4.402	1876	0.089	977
	Total		21397150				100	

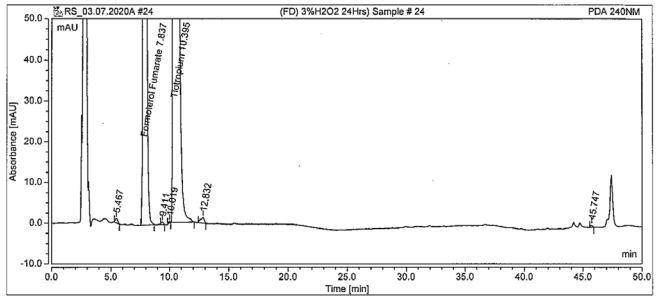
Figure 5f. Chromatogram of acid degraded sample solution

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 0.45 µm nylon membrane filters can be used, and it is



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.821	Formoterol Fumarate	5900413	BMB	0.753	352475	27.534	999
2	9.397		6766	BMB	0.905	635	0.032	901
3	10.381	Tiotropium	15504439	BMB	1.000	768777	72.352	1000
4	12.624		13516	BMB	1.216	981	0.063	945
5	31.984		4167	BMB	3.081	325	0.019	747
	Total		21429302				100	

Figure 5g. Chromatogram of base degraded sample solution



No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	5.467		16460	BMB	0.526	1139	0.080	996
2	7.837	Formoterol Fumarate	5527781	BMB	0.754	370706	26.730	999
3	9.411		7501	BMB	0.905	698	0.036	893
4	10.019		4729	BMB	0.964	489	0.023	810
5	10.395	Tiotropium	15088954	BMB	1.000	750783	72.964	1000
6	12.832		28505	BMB	1.234	1478	0.138	872
7	45.747		6163	BMB	4.401	572	0.030	822
	Total		20680091				100	

Figure 5h. Chromatogram of hydrogen peroxide degraded sample solution

recommended to discard the 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.

The 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. The cumulative% RSD of impurity results (above LOQ level) obtained using stored sample solutions should not be more than 5.0.

Table 6. Forced degradation

Sr. no.	Degradation condition	Degrading 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	0.189	0.575	1000	999	Passes
2	Photolytic	1.2 million lux hours; 200 watt hrs./m^2	For 7 days	0.086	0.336	1000	999	Passes
3	Humidity	40°C/75% RH	For 7 days	0.092	0.179	1000	999	Passes
4	Acid	0.01N HCl	For 1 hr at RT	0.196	0.597	1000	999	Passes
5	Base	0.001N NaOH	For 5 min at RT	0.098	0.177	1000	999	Passes
6	Peroxide	3% H_2O_2	For 24 hr at RT	0.206	0.458	1000	999	Passes

TIO: Tiotropium, FF: Formoterol fumarate, NA: Not applicable, RH: Relative humidity, Sr. no: Serial number

Table 7. Linearity

Linearity FF				Linearity TIO			
Linearity level	Conc. (%)	Conc. (ppm)	Area	Linearity level	Conc. (%)	Conc. (ppm)	Area
1	LOQ	0.010	1386	1	LOQ	0.015	3489
2	20	0.097	13138	2	20	0.145	35978
3	50	0.243	34008	3	50	0.363	83211
4	80	0.388	53406	4	80	0.581	135688
5	100	0.485	69004	5	100	0.726	167367
6	120	0.582	79151	6	120	0.872	195637
7	150	0.728	101903	7	150	1.089	245102
Slope		139302.5901		Slope		224043.3645	
Intercept		-122.4944		Intercept		2446.2293	
Correlation coefficient		1.000		Correlation coefficient		1.000	

FF: Formoterol fumarate, LOQ: Limit of quantification, Conc.: Concentration

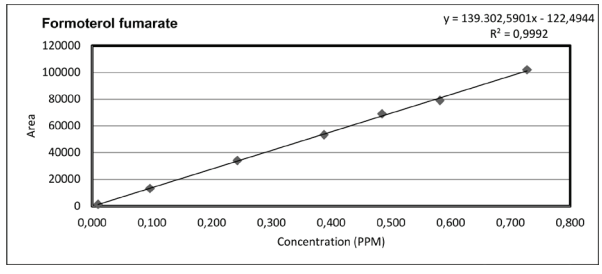


Figure 6. Linearity of formoterol fumarate

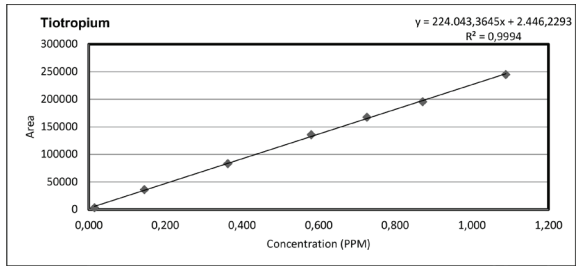


Figure 7. Linearity of tiotropium

Table 8. Accuracy

Accuracy at LOQ Level FF				Accuracy at LOQ level TIO			
Preparation	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Preparation	Amount added (ppm)	Amount recovered (ppm)	% Recovery
1	0.0100	0.0094	94.0	1	0.0148	0.0150	101.4
2	0.0100	0.0099	99.0	2	0.0148	0.0155	104.7
3	0.0100	0.0094	94.0	3	0.0148	0.0153	103.4
4	0.0100	0.0095	95.0	4	0.0148	0.0151	102.0
5	0.0100	0.0098	98.0	5	0.0148	0.0147	99.3
6	0.0100	0.0089	89.0	6	0.0148	0.0148	100.0
Average			94.8	Average			101.8
SD			3.5449	SD			2.0327
% RSD			3.74	% RSD			2.00

Inj. no.	FF accuracy 50% level			FF accuracy 100% level			TIO accuracy 150% Level		
	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Amount added (ppm)	Amount recovered (ppm)	% Recovery
1	0.2488	0.2500	100.5	0.4976	0.5035	101.2	0.7464	0.7305	97.9
2	0.2488	0.2408	96.8	0.4976	0.4822	96.9	0.7464	0.7330	98.2
3	0.2488	0.2360	94.9	0.4976	0.4903	98.5	0.7464	0.7502	100.5
Average			97.4			98.9			98.9
SD			2.8478			2.1733			1.4224
% RSD			2.92			2.2			1.44

Inj. no.	TIO accuracy 50% level			TIO accuracy 100% level			TIO accuracy 150% Level		
	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Amount added (ppm)	Amount recovered (ppm)	% Recovery
1	0.3710	0.3673	99.0	0.7419	0.7532	101.5	1.1129	1.0847	97.5
2	0.3710	0.3816	102.9	0.7419	0.7298	98.4	1.1129	1.1073	99.5
3	0.3710	0.3762	101.4	0.7419	0.7371	99.4	1.1129	1.1067	99.4
Average			101.1			99.8			98.8
SD			1.9672			1.5822			1.1269
% RSD			1.95			1.59			1.14

SD: Standard deviation, RSD: Relative standard deviation, LOQ: Limit of quantification, TIO: Tiotropium, FF: Formoterol fumarate

Table 9. Filter validation

Sample solution	% Impurity		Absolute % difference	
	% Single maximum impurity	% Total impurity	% Single maximum impurity	% Total impurity
Centrifuged	0.105	0.201	NA	NA
0 mL discarded	0.106	0.343	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

NA: Not applicable

The solution is considered stable, until 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).

Table 10. Robustness

Parameters	Wavelength (nm) (+/-3)		Flow rate (mL/min) (+/0.1 mL/min)		Column temperature (°C) (+/- 5°C)		Gradient composition (+/- 5%)		Buffer pH (+/- 0.2)	
	237	243	0.9	1.1	25°C	35°C	-5%	+5%	3.0	3.4
Similarity factor	98.5	96.9	100.1	100.7	99.0	99.3	98.6	99.3	99.6	99.0
T.F.	1.2	1.2	1.2	1.2	1.3	1.3	1.3	1.3	1.3	1.3
NTP	10063	9990	10951	8797	10828	9817	10952	10082	9638	9110

T.F.: Tailing factor, NTP: Number of therotical plates, °C: Degree celsius

Table 11. Solution stability

Stability data for standard solution					Stability data for sample solution						
Time point	% 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

TIO: Tiotropium, SD: Standard deviation, RSD: Relative standard deviation, Avg: Average

CONCLUSION

The recommended analytical method for the related substance determination of Tiomate Transcaps® DPI is simple, robust, selective, specific and precise. It also demonstrates the study of degradation pattern; therefore, can be used for quality control testing, routine analysis and for stability studies.

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