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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'DAKİ İLGİLİ MADDELERİN KANTİTATİF TAYİNİ

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, 30ºC 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
Astım, bronkospazm, kronik bronşit, amfizem ve kronik obstrüktif akciğer hastalıklarının (KOAH) tedavisinde kullanılan kuru toz inhaler dozaj formunda 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 geliştirmeyi hedefleyin, ancak bunun için en önemlisi hassas, basit, sağlam ve onaylanmış bir yönteme sahip olmaktır, bu nedenle yeni bir RP-HPLC Formoterol fumarat ve Formoterol fumarat dihidrat içindeki Tiotropium ve inhalasyon için Tiotropium bromide kuru tozdaki ilgili maddelerin belirlenmesi için yöntem geliştirilmiştir. Kromatografik ayırma, tampon çözeltisi pH 3.2'nin mobil faz A olarak ve asetonitrilin 1.0 mLmin-[1] akış hızında, 30°C kolon sıcaklığında ve 240nm dalga boyunda PDA detektöründe ve Hypersil BDS C18 kolonunda (250) mobil faz B olarak kullanıldığı bir gradyan elüsyonu kullanır. 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-(1*RS*)-1-hydroxy-2-(1*RS*)-2(4methoxyphenyl)1methylethylaminoethyl 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]

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 (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-azoniatricyclo3.3.1.02,4nonanebromidemonohydrate with molecular formula $C_{19}H_{22}BrNO_4S_2\cdot H_2O$ 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, 17–24], also in plasma, urine and biological samples [25, 26]. TIO has been determined with either FF [27–29] or ciclesonide 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) 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 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,

Calculation,

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

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

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 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 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 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,

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 0.008ppm and LOQ is 0.024 ppm and reported LOQ = 0.015ppm 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 0.007ppm 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 0.45 µm 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 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%

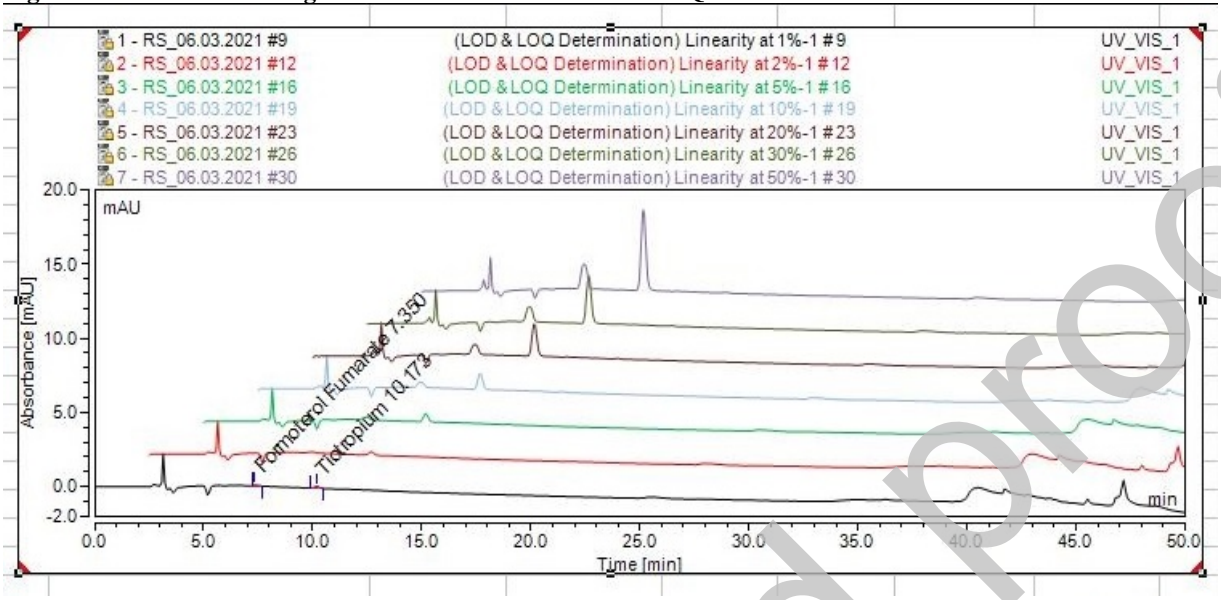


Fig 2 - LOD & LOQ determination of FF

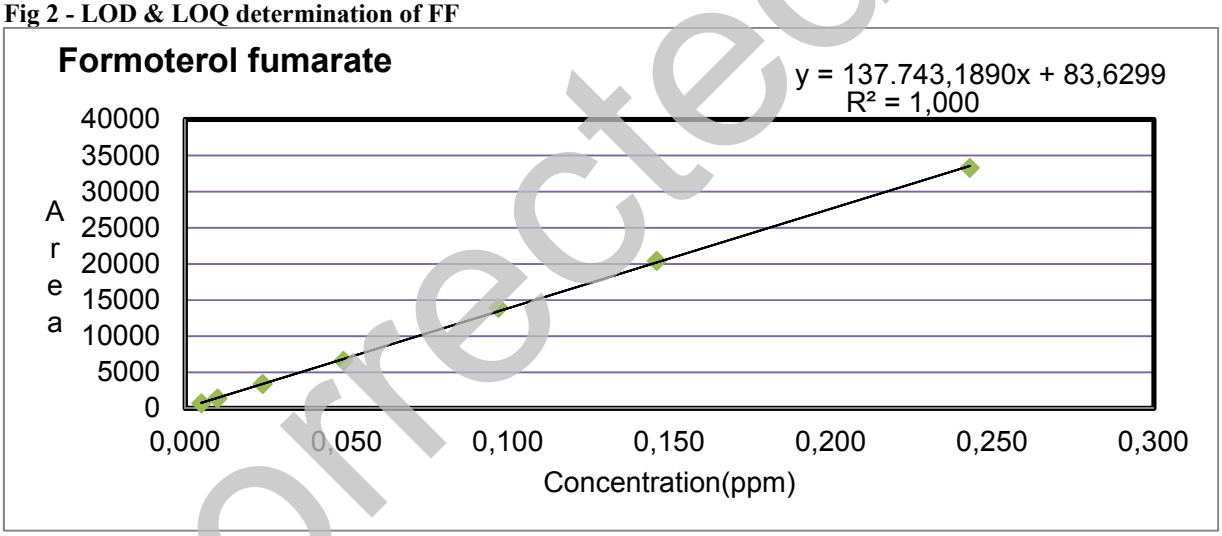


Fig 3 - LOD & LOQ determination of TIO

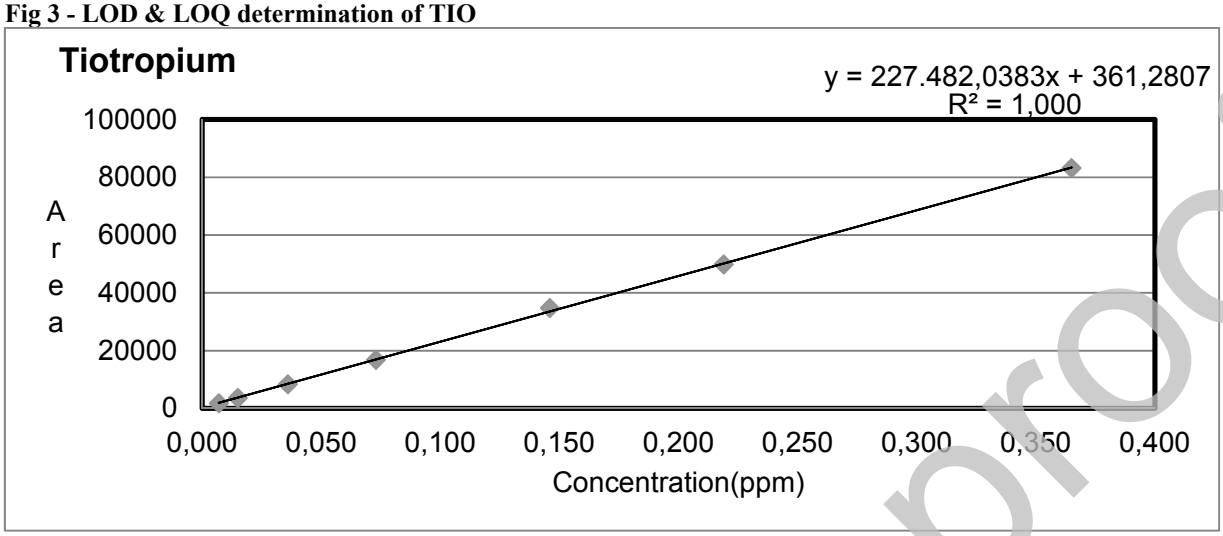
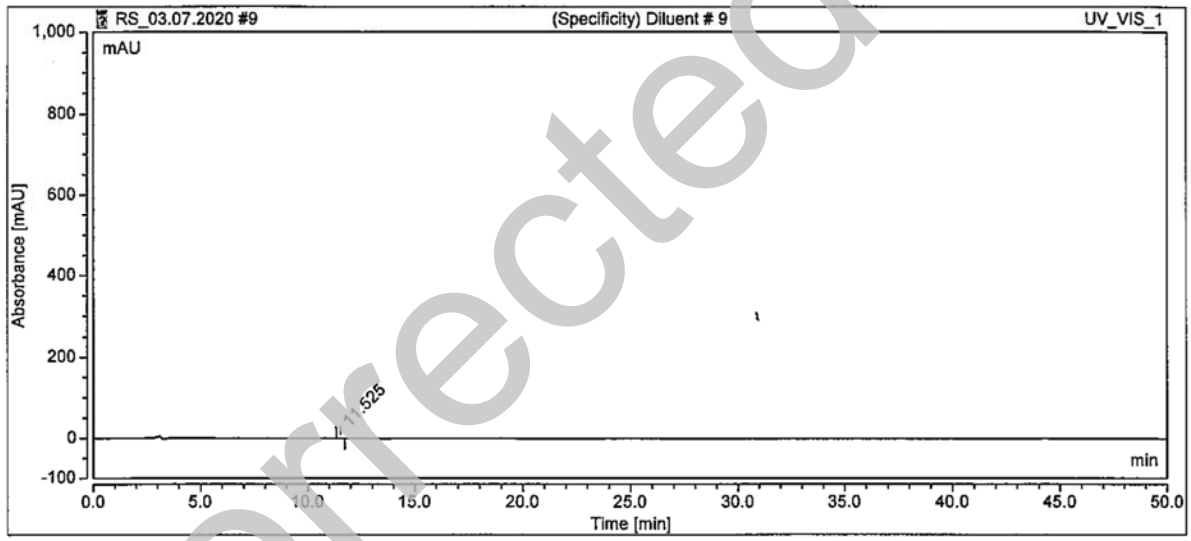
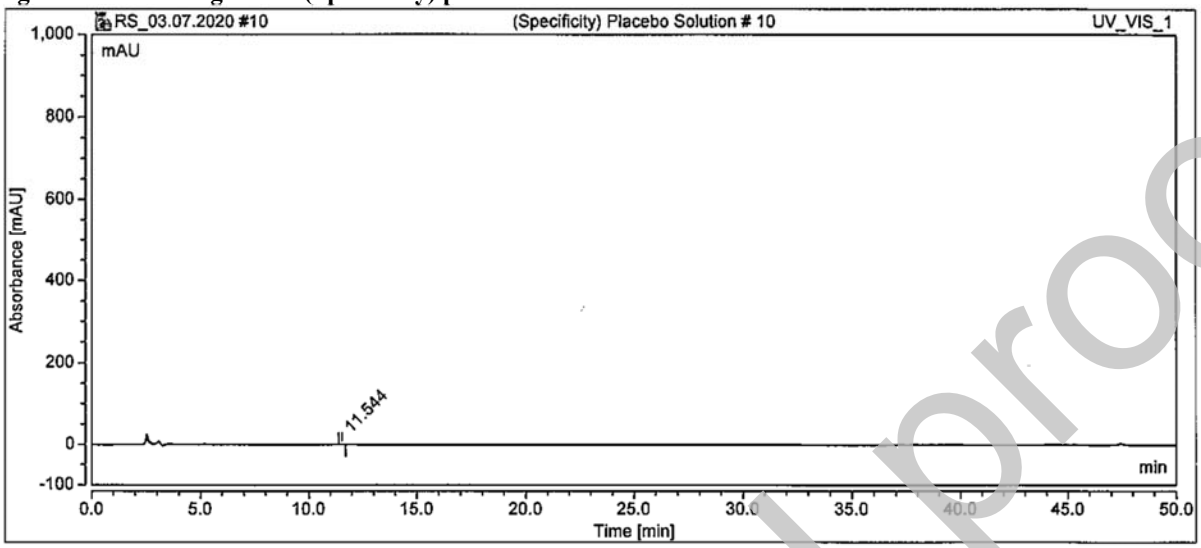


Fig. 4a – Chromatogram of (Specificity) Diluent



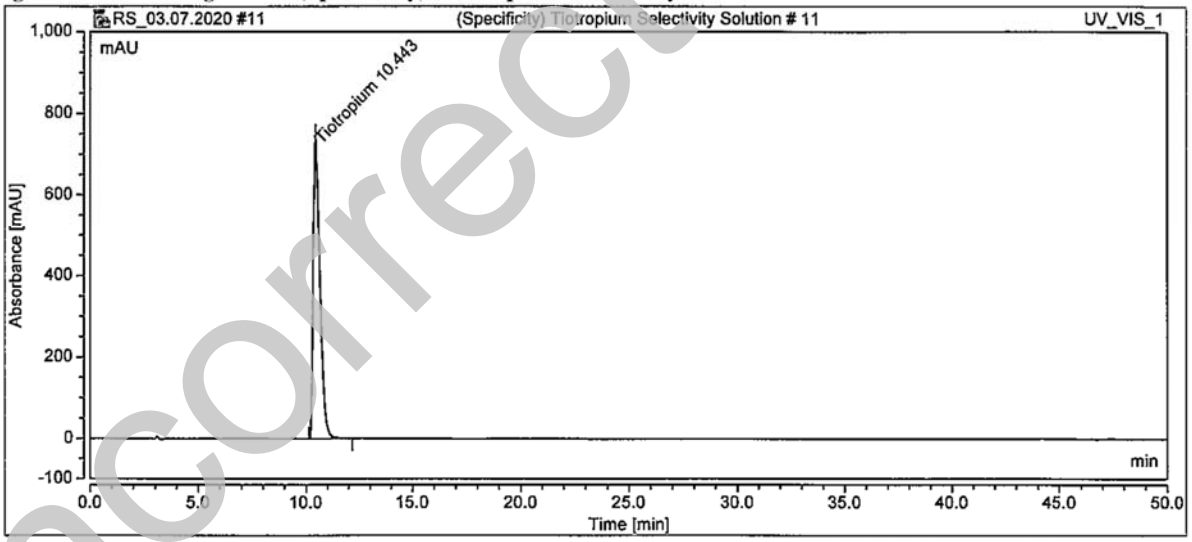
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	11.525		2232	BMB	n.a.	175	100.000	730
	Total		2232				100	

Fig. 4b – Chromatogram of (Specificity) placebo solution



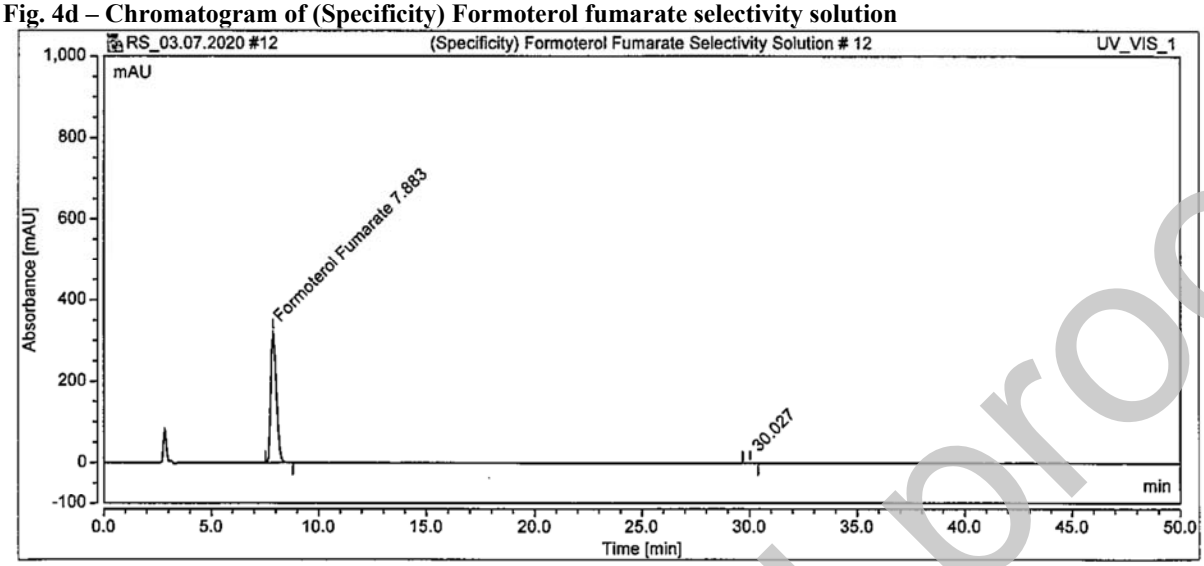
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	11.544		1532	BMB	n.a.	138	100.000	625
	Total		1532				100	

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



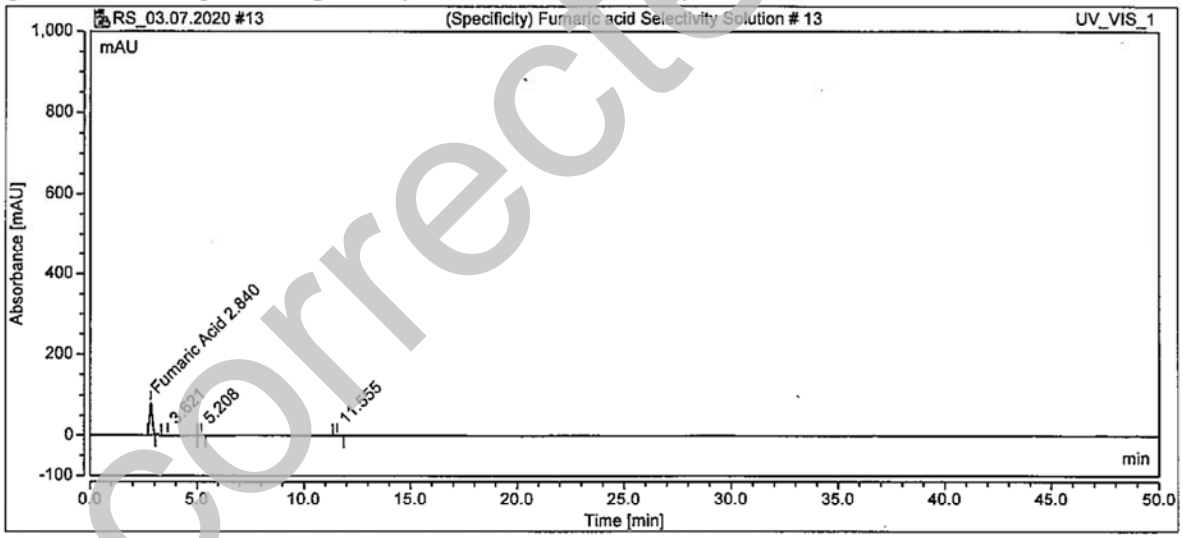
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	10.443	Tiotropium	16214683	BMB	1.000	744630	100.000	1000
	Total		16214683				100	

Fig. 4d – Chromatogram of (Specificity) Formoterol fumarate selectivity solution



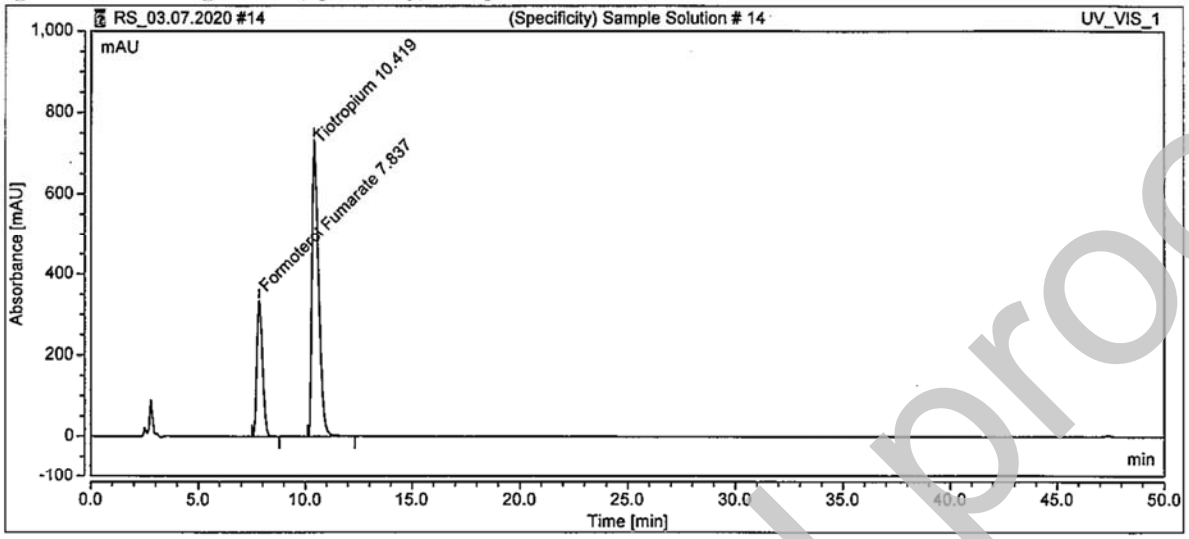
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.883	Formoterol Fumarate	5864148	BMB	n.a.	322421	99.855	1000
2	30.027		8490	BMB	n.a.	389	0.145	800
	Total		5872637				100	

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



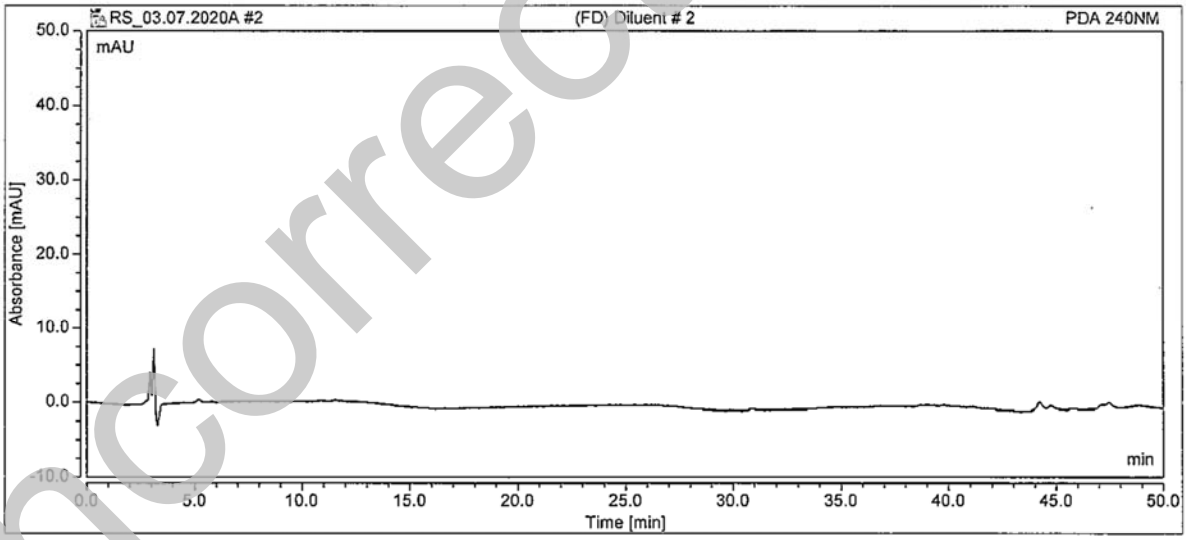
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	2.840	Fumaric Acid	632702	BMB	n.a.	79544	86.926	1000
2	3.621		87584	BMB	n.a.	1629	12.033	990
3	5.208		4370	BMB	n.a.	415	0.600	934
4	11.555		3204	BMB	n.a.	195	0.440	650
	Total		727861				100	

Fig. 4f – Chromatogram of (Specificity) Sample solution



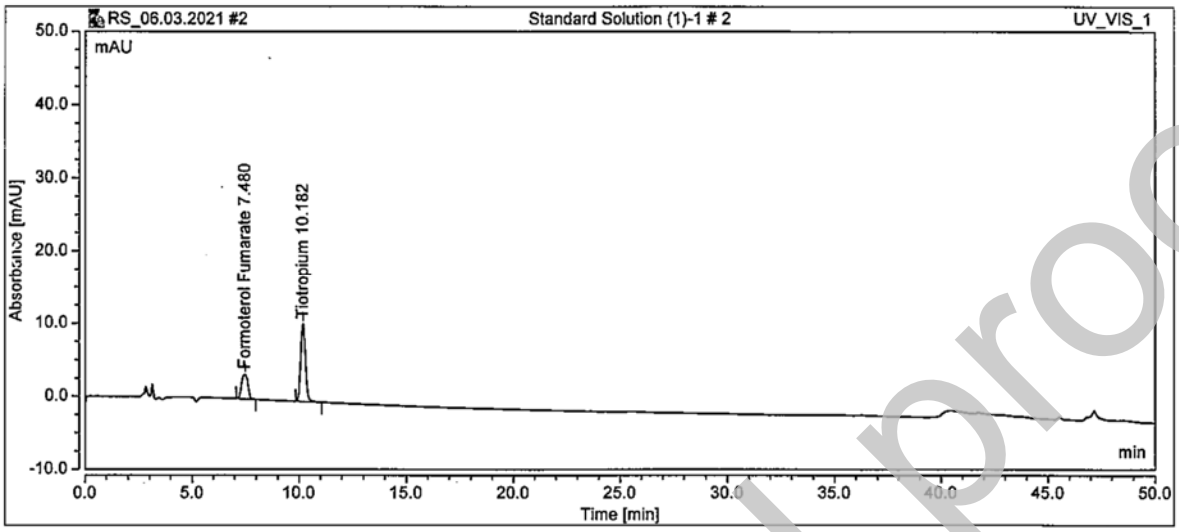
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.837	Formoterol Fumarate	6240826	BMB	0.752	333107	28.762	999
2	10.419	Tiotropium	15457260	BMB	1.000	734295	71.238	1000
	Total		21698086				100	

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	

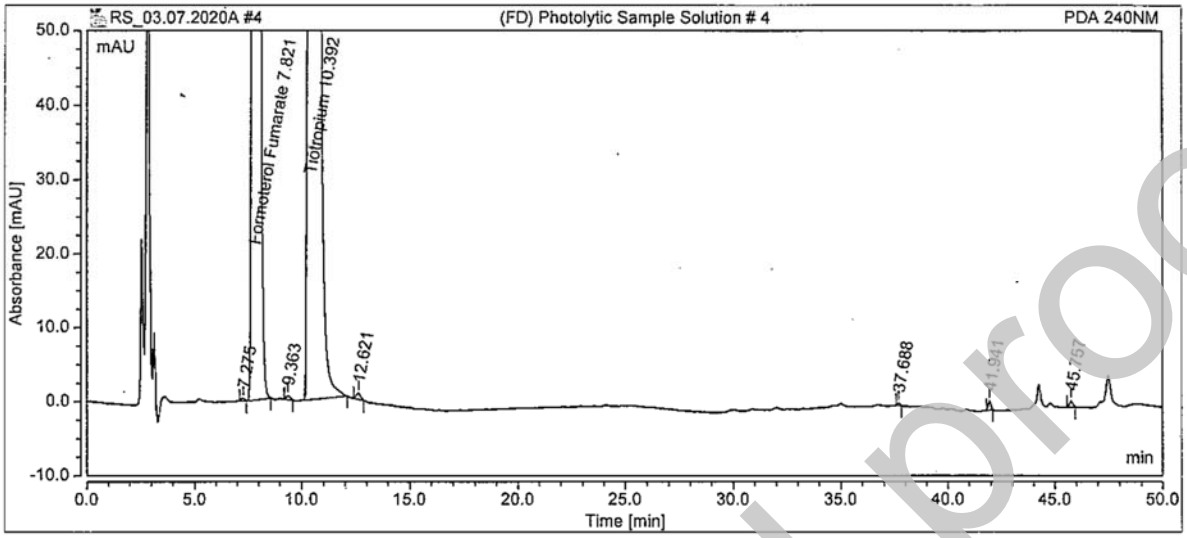
Fig 5b – Typical chromatogram of Standard Solution



No	Peak Name	Ret.Time 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	

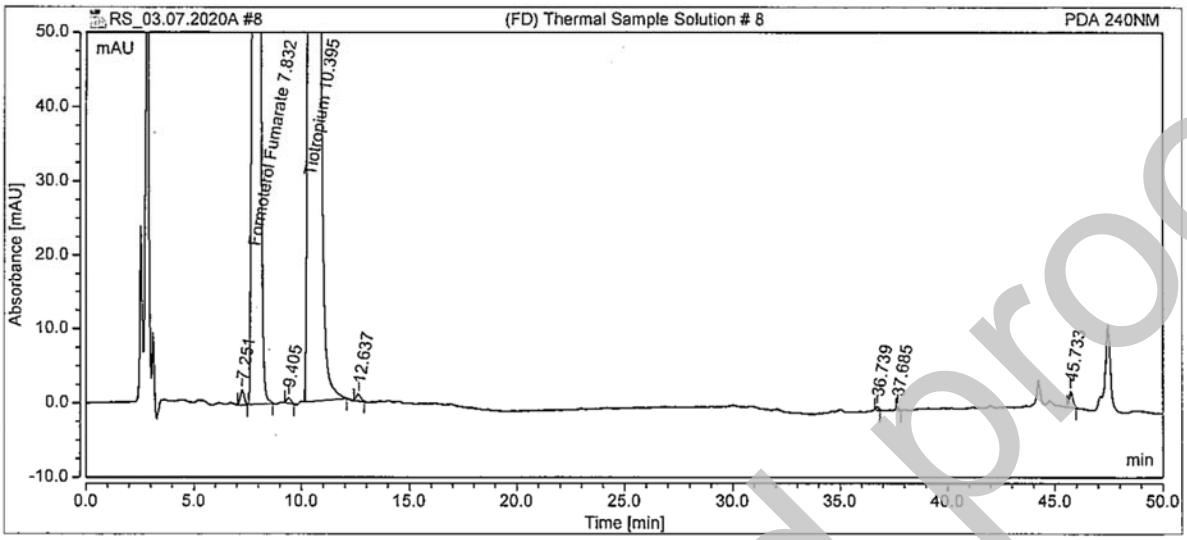
No	Peak Name	Ret.Time min	Height µAU	Resolution (USP)	Theoretical Plates	Asymmetry
1	Formoterol Fumarate	7.480	3319	n.a.	3905	1.0
2	Tiotropium	10.182	10631	6.2	10526	1.1

Fig 5c – Chromatogram of Photolytic degraded sample solution



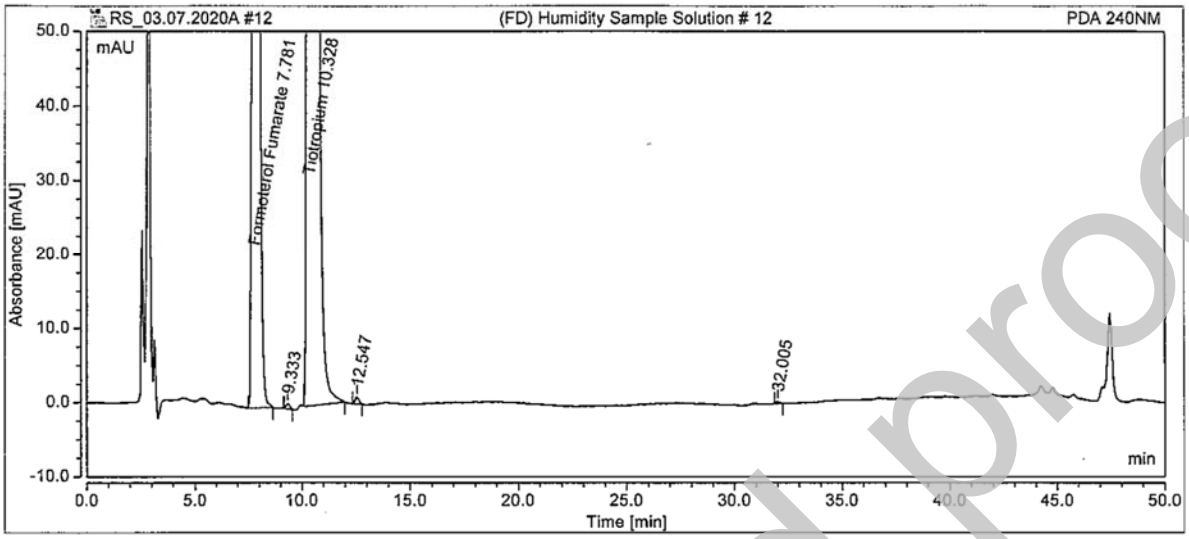
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.275		3981	BMB	0.700	375	0.018	940
2	7.821	Formoterol Fumarate	6121462	BMB	0.753	330534	27.975	999
3	9.363		7282	BMB	0.901	570	0.033	918
4	10.392	Tiotropium	15713808	BMB	1.000	777249	71.812	1000
5	12.621		11966	BMB	1.215	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		21881883				100	

Fig 5d – Chromatogram of Thermal degraded sample solution



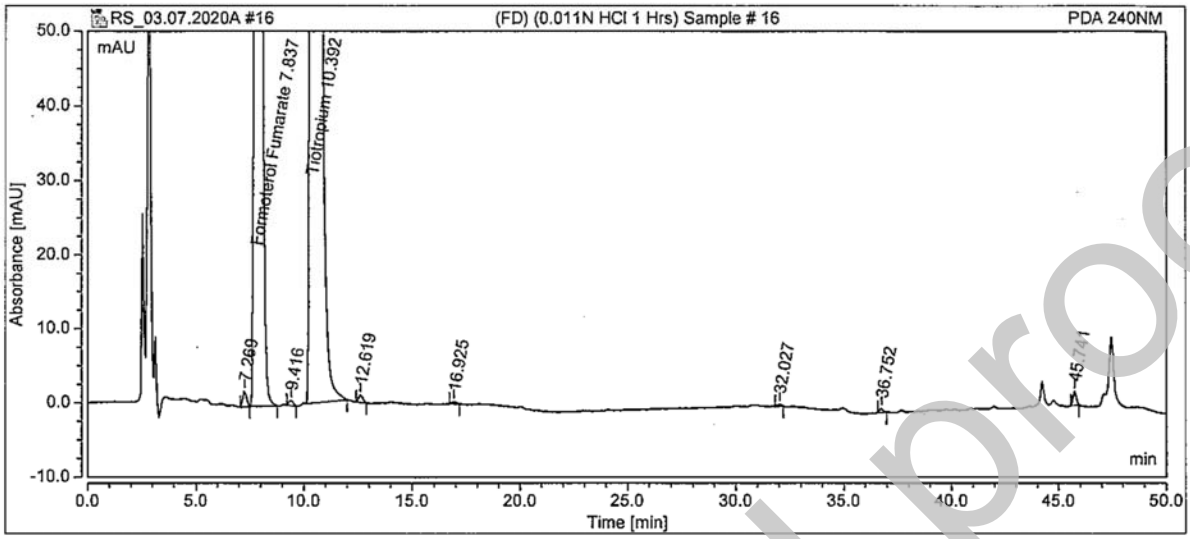
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.251		26342	BMB	0.698	2049	0.122	999
2	7.832	Formoterol Fumarate	5875643	BMB	0.753	358268	27.134	999
3	9.405		9267	BMB	0.905	774	0.043	929
4	10.395	Tiotropium	15698262	BMB	1.000	781496	72.495	1000
5	12.637		13434	BMB	1.216	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	

Fig 5e –Chromatogram of Humidity degraded sample solution



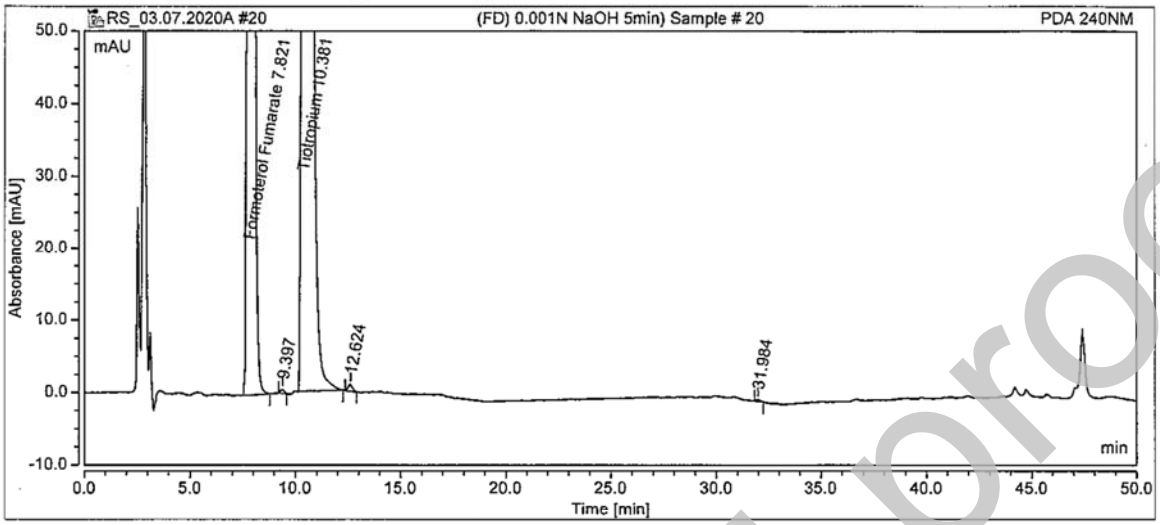
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.781	Formoterol Fumarate	5737688	BMB	0.753	352290	27.170	999
2	9.333		8407	BMB	0.904	703	0.040	876
3	10.328	Tiotropium	15355160	BMB	1.000	776204	72.713	1000
4	12.547		12603	BMB	1.215	963	0.060	910
5	32.005		3551	BMB	3.099	281	0.017	659
	Total		21117409				100	

Fig 5f –Chromatogram of Acid degraded sample solution



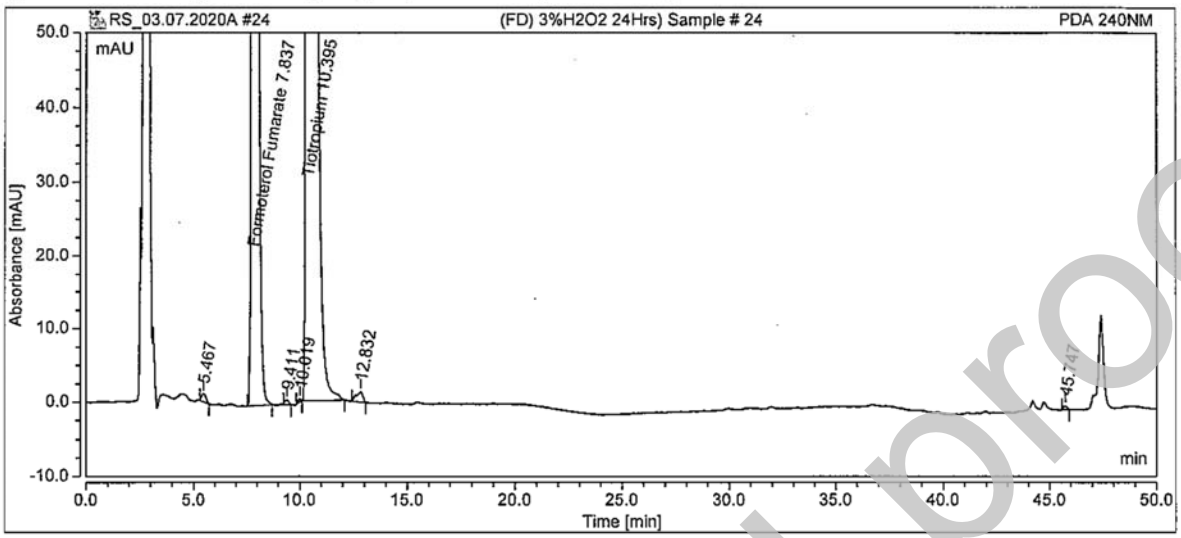
No	Ret. Time min	Peak Name	Area µAU*sec	Peak Type	RRT	Height µAU	Area %	Match
1	7.269		27152	BMB	0.700	2129	0.127	998
2	7.837	Formoterol Fumarate	5848700	BMB	0.754	352096	27.334	999
3	9.416		9554	BMB	0.906	744	0.045	843
4	10.392	Tiotropium	15465666	BMB	1.000	775429	72.279	1000
5	12.619		12987	BMB	1.214	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		2139715				100	

Fig 5g –Chromatogram of Base degraded sample solution



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	

Fig 5h – Chromatogram of Hydrogen peroxide 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	15038954	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		20630091				100	

Fig 6 - Linearity of FF

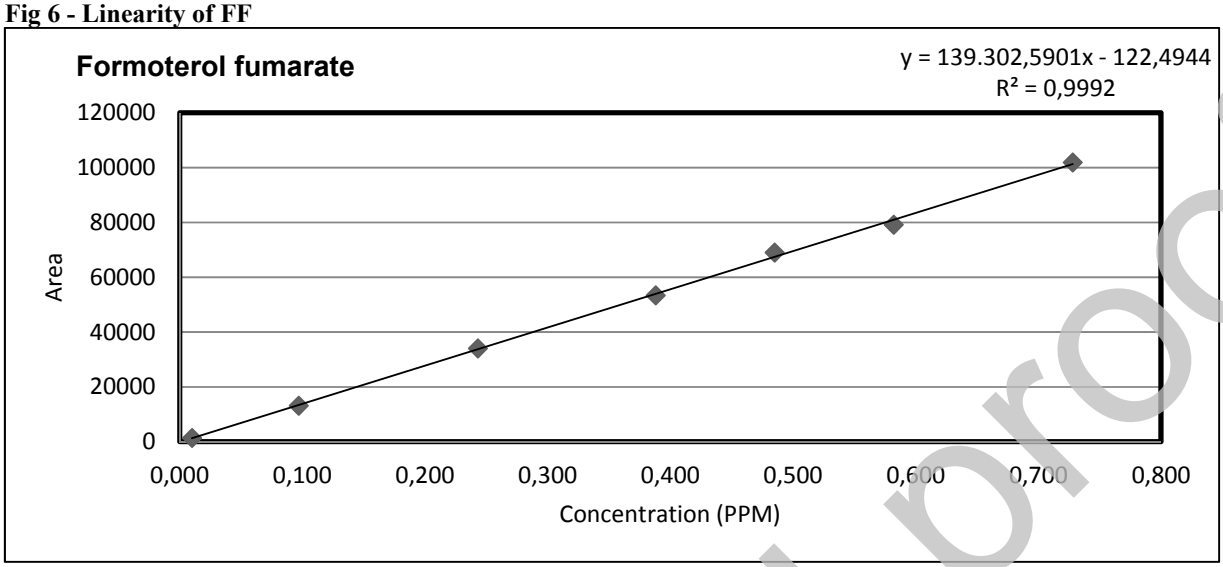


Fig 7 - Linearity of TIO

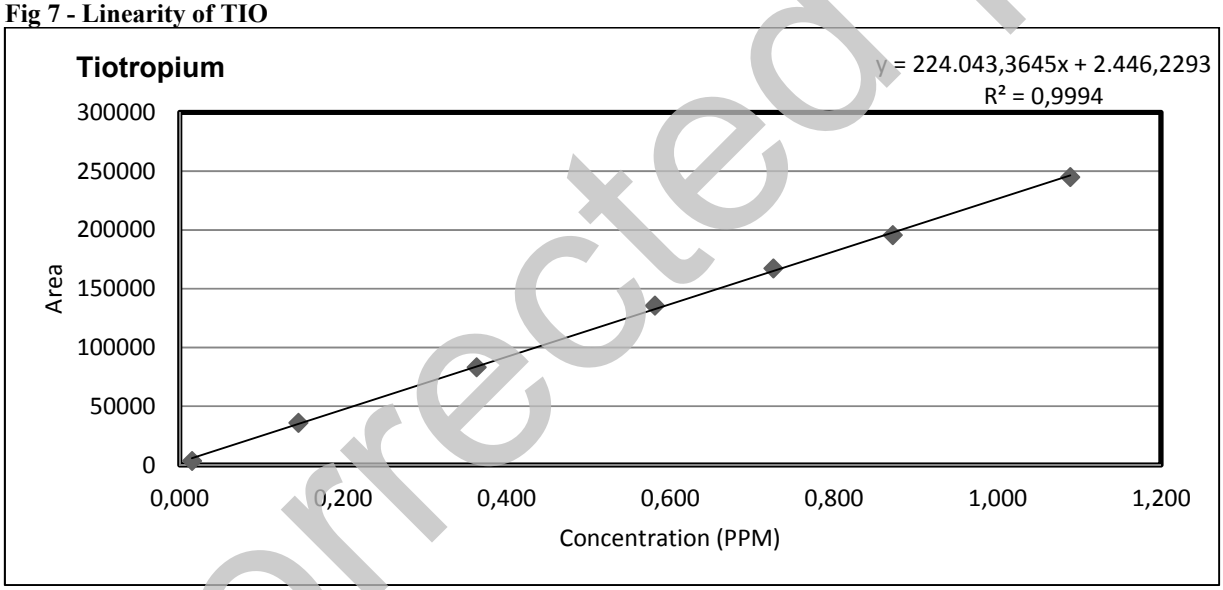


Table 1: System suitability & System Precision

System suitability					**System precision**	
Injection	**Area**	**Similarity factor**	**Tailing factor**	**Theoretical plates**	**Average area of 6 replicate standard injections**	140531
Standard solution -1	140777	100.5	1.1	10513	**Standard deviation**	1615.5497
Standard solution - 2	141361		NA	NA	**% RSD**	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	0.142	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**

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. % Single maximum impurity (above LOQ Level) = 0.093 % total impurity = 0.167	1000	999	Passes

Table 5: 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 5min. at RT	0.098	0.177	1000	999	Passes
6	**Peroxide**	3% H2O2	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

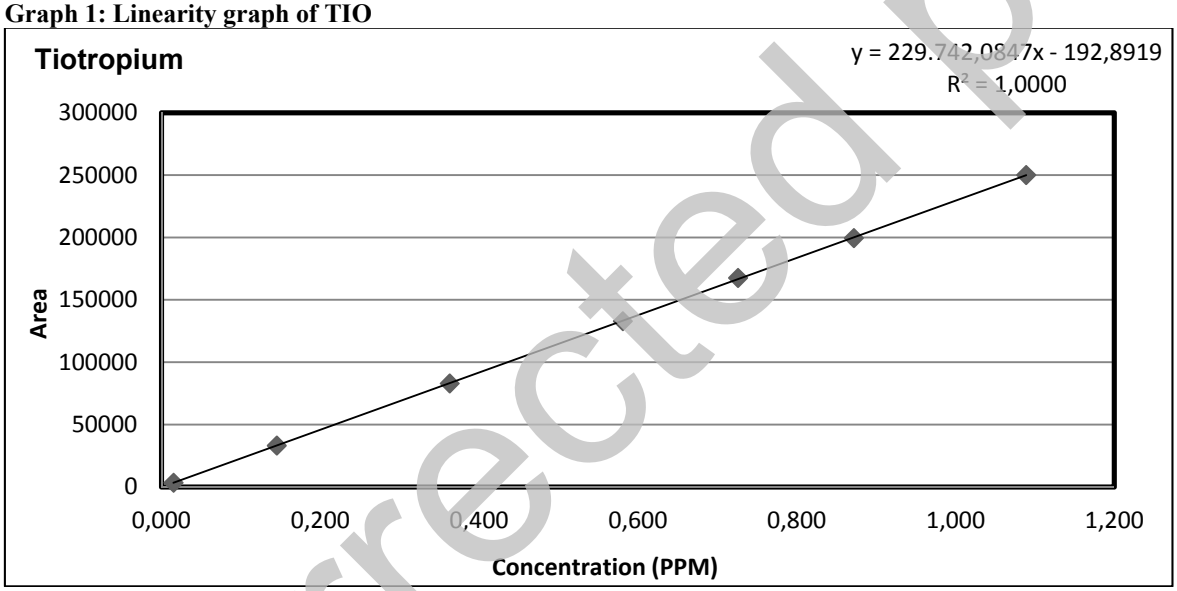


Table 7: Accuracy

	Accuracy at LOQ Level		
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			**101.4**

SD	5.2576
% RSD	**5.19**

Inj. No.	Accuracy 50% Level			Accuracy 100% Level			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	0.3738	0.3821	102.2	0.7475	0.7610	101.8	1.1213	1.1257	100.4
2	0.3738	0.3847	102.9	0.7475	0.7652	102.4	1.1213	1.1260	100.4
3	0.3738	0.3914	104.7	0.7475	0.7641	102.2	1.1213	1.1292	100.7
Average			**103.3**			**102.1**			**100.5**
STDEV			1.2897			0.3055			0.1732
% RSD			**1.25**			**0.30**			**0.17**

Table 8: 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

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)	
	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

Table 10: 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**