HPLC Determination of Indacaterol Maleate in Pharmaceutical Preparations Adopting Ultraviolet and Fluorescence Detection

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ABSTRACT

Sensitive and rapid HPLC procedure was developed for determination of Indacaterol Maleate (IND) in bulk powder and capsules using Ultraviolet and fluorescence detection. The chromatographic separation was performed on C18 column as a stationary phase, and the mobile phase consisted of acetonitrile: 5mM acid hydrogen orthophosphate containing 0.3 % triethylamine (TEA) in ratio of (40: 60% v/v) adopting both UV detection and fluorescent detection adjusted to pH 3.0 using 0.02 M orthophosphoric acid (OPA) and was passed at flow rate 1.0 mL /min. The UV detection was adjusted at 259 nm where dexamethasone was used as internal standard or fluorescence detection was at 421 nm after excitation at 258 nm where Cyproheptadine was used as internal standard. The developed method was validated according to ICH guidelines in terms of linearity, lower limit of quantification (LOQ), lower limit of detection (LOD), precision and accuracy. The absorbance-concentration plot was rectilinear over the range 2.0 - 20.0 µg /mL with a lower detection limit (LOD) of 0.116 µg /mL and lower quantification limit (LOQ) of 0.352 µg /mL adopting UV detection. Meanwhile, the fluorescence-concentration plot was rectilinear over the range of 0.05 – 5.0 µg /mL with LOD of 8.6×10^{-3} µg /mL and LOQ of 26.1×10^{-3} µg /mL adopting fluorescent detection The proposed method was rapid (elution time didn't exceed 5 min) and reproducible (R.S.D. < 2.0%). The proposed method was applied successfully for the determination of Indacaterol maleate in capsule dosage form. The developed method adopting fluorescence detector is 50 times more sensitive than Ultraviolet method.

Keywords: - Indacaterol Maleate (IND); HPLC; Assay, Capsules, Ultraviolet, Fluorescence detection

1. Introduction

Indacaterol (IND) is designed chemically as 5-{(1R)-2-[(5,6-diethyl-2,3-dihydro-1H-inden-2-yl)amino]-1-hydroxyethyl}-8-hydroxy-2(1H)-quinolinone maleate[1] (Fig. 1). It is a new, long-acting β_2-agonist bronchodilator used for maintenance treatment of airflow problems in patients with chronic obstructive pulmonary disease[2] (COPD). Two HPLC/MS methods [3,4] were developed for detection of IND in biological fluids. Spectrophotometric and spectrofluorimetric methods were developed in our laboratories for its determination in pure form and capsule dosage forms[5]. To the best of our knowledge no further simple chromatographic methods have been reported up till now for determination of IND either in pure form or in capsules.

The aim of the work

There is an increasing drive towards achieving time and cost effective HPLC analysis of IND to be applied in quality control laboratories. In this work a valid, sensitive and accurate, RP-HPLC method was developed for determination of IND in pure form and capsules. Different chromatographic parameters were carefully studied to optimize the analytical determination of the studied compound. The proposed method adopting fluorescence detector is 50 times more sensitive than Ultraviolet detector.

2. Experimental

2.1 Materials and Chemicals

All chemicals used were of Analytical Reagent grade, and the solvents were of HPLC grade.

- Indacaterol maleate (IND) was kindly provided by Wuhan Vanz Pharm Inc. High technology Industry Park, Wuhan Economic & Technology Development Zone, Wuhan, 430056, China (Purity 99 %.).They were used as received without further purification.

- Onbrez® capsules contain 150 µg Indacaterol Maleate (IND), manufactured by Novartis Pharma Inc., East Hanover, USA. Batch no. 143984 were obtained from local pharmacy.
- Acetonitrile, Methanol and acid hydrogen phosphate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).
- Orthophosphoric acid (OPA) Triethylamine (TEA) was obtained from Prolabo (Paris, France).

2.2 Apparatus

Separation was performed with shimadzu™ LC-20A series chromatograph equipped with a 20 µL Rheodyne injector valve and a SPD-20A UV detector operated at 259 nm, and RF-10AXL fluorescence detector operated at (λex/em: 258/421 nm). LC workstation (Nishinokyo- Kuwabaracho, Nakagyo- Ku,Kyoto, Japan), Total Chrom Workstation (Massachusetts, USA) was applied for data collecting and processing. Mobile phase was degassed using Merck solvent L-7612 degasser. A Consort P-901 pH-meter was used for pH measurements.

2.3 Columns and mobile phases

A Hibar® C18, pre-packed column RT (150 mm × 4.6 mm ID, Lichrosorb® RP-18 (5 µm particle size) was used as a stationary phase. The column was operated at ambient temperature. The mobile phase consisted of acetonitrile: 10 Mm acid hydrogen phosphate buffer containing 0.3 % TEA [40: 60] for both ultraviolet and fluorescence detection. The pH of the mobile phase was adjusted after mixing to 3.0 using 0.02 M OPA and was pumped at flow rate of 1 mL/ min. The mobile phase was shaken on an ultrasonic bath for 5 min and was filtered through a 0.45-µm membrane filter (Millipore, Ireland).

2.4 Standard stock and working solutions:

Stock solution of 100 µg/mL of IND was prepared by dissolving 10.0 mg of IND into 100 mL measuring flask containing methanol. The mobile phase was used for further dilution of standard solutions to reach the required concentration range of 2.0-20 µg/mL for ultraviolet detector and 0.05-5.0 µg/mL for a fluorescence detector. The standard solutions were found to be stable for at least one week when kept in the refrigerator.

2.4 .1 General procedures and calibration graphs

To a set of 10 mL volumetric flasks, increasing volumes of the standard solution of IND were quantitatively transferred so as to give solutions within the working concentration range of 2-20 and 0.05-5.0 µg/mL for ultraviolet and fluorescence detection, respectively. The flasks were further diluted to 10.0 mL with the mobile phase. Twenty microliter aliquots were injected (in triplicate) at ambient temperature (25 ºC) and eluted with the specified mobile phase under the reported chromatographic conditions and the calibration curves were constructed by plotting the peak area ratio [drug/I.S.] against the final concentration of IND (µg/mL). Alternatively, the corresponding regression equations were derived.

2.4.2 Analysis of the studied drug in its capsules

The contents of ten capsules were emptied and mixed well. A weighed quantity of the powder equivalent to 1.5 mg of IND was transferred into a small conical flask and extracted three successive times each with 30 mL of methanol. The extracts were collected, filtered and transferred quantitively into a 100 mL volumetric flask and completed to the volume with the same solvent. All samples were filtered through 0.45 µm sample filters (RC25, Sartorius AG, Gottingen, Germany) prior to injection into the HPLC system. Three different concentrations covering the working concentration range for both detection methods were transferred into two sets of 10 mL volumetric flasks and the procedures for the calibration graph were followed as described under **"Construction of calibration curves"**. The nominal content of the capsules was determined using the corresponding regression equation.

3. Results & Discussion

3.1 Method development

The separation was achieved using a mobile phase of pH 3.0 consisting of acetonitrile: acid hydrogen phosphate buffer containing 0.3 % TEA in a ratio of either [40: 60%, v/v] for both detection modes. Determination of IND within 5 min run time was achieved by the proposed method; figure 2, and 3 represent the obtained chromatogram of IND using ultraviolet and fluorescence detection, respectively.

The most appropriate chromatographic system was developed upon studying the effect of many experimental parameters of the chromatographic system. The USP guidelines[6] describes these parameters in terms of number of theoretical plates, and tailing factor.

3.1.1The Stationary phase

Two different columns were investigated: Cyano column (250 x 4.6 mm i.d., 5 µm particle size), and Hibar® C18, pre-packed column (150 mm×4.6 mm ID., 5-µm particle size). Experimental studies revealed that the use of C18 column used gives better, symmetrical and well defined peak.

3.1.2 The mobile phase

3.1.2 .a Effect of pH

The mobile phase pH was altered using increasing volumes of 0.3 % triethylamine (TEA) over a specified range of 3.0 to 6.0. The retention time of IND was not changed after increasing the pH value up to pH 6.0 as IND will be completely ionized over the investigated pH range (pKa value of IND = 8.5 and 9.7)[7]. At pH 6.0, the efficiency of the peak was not suitable for measurements as indicated by the values of N, and tailing factor. For both detectors, a pH value of 3.0 was found to be optimum as it offers resolving and quantitation of both IND drug, and the internal standard in a short run coupled with a good selectivity, peaks efficiency and peak symmetry as presented in table 1.

3.1.2 .b Ratio of Mobile Phase Components

As shown in table 1, increasing ratios of ACN: 0.05 M acid hydrogen phosphate buffer were investigated over the range from 30: 70 to 70: 30. It was found that increasing ratio of buffer to acetonitrile (70:30, v/v) retained IND on the column for more than 8 min. Meanwhile, upon increasing acetonitrile content versus acid hydrogen phosphate (70:30, v/v), it was noticed that the drug was un-retained as the retention time was greatly decreased and the drug peak was overlapped with solvent front.

The sensitivity parameter based on the peak area of the drug and the peak efficiency based on number of theoretical plates (N) were the basis of choosing the optimum acetonitrile ratio in the mobile phase. Thus, the optimum ratio was found to be (40: 60%, v/v) acetonitrile: acid hydrogen phosphate buffer. It provides the best sensitivity, resolution and reasonable run time for both detectors.

3.1.2. c Type of organic modifier:

Different organic modifiers including methanol and n-propanol were investigated as alternates for acetonitrile. Replacement of acetonitrile with methanol retained IND on the column for more than 8 min with lower sensitivity. Also, the replacement of phosphate buffer with water resulted in decreased peak efficiency as revealed by lower number of theoretical plates, and broadening of IND peak. Thus, acetonitrile and sodium phosphate were the ideal selection for the chromatographic separation.

3.1.2 .d The Flow rate

The effect of flow rate was studied to optimize the chromatographic efficiency of the proposed method and improve the resolution of the eluted peaks. The flow rate was changed over the range of 0.2-1.2 mL/min and a flow rate of 1 mL/min was the optimum for good separation in a reasonable time adopting both detectors. The results are shown in Table I

3.1.2. e The Choice of the Internal Standard:

The use of internal standard is very important for providing a well-developed accurate and precise HPLC method. Different drugs such as dexamethasone, rouvastatin, ezetimibe, cyproheptadine, terbutaline, and ciprofloxacin were investigated as possible internal standards. Dexamethasone and cyproheptadine were the best internal standard that provides excellent separation of its peak from the intact drug for both UV and fluorescent detectors, respectively. While, other drugs under investigations gave overlapping peaks with the drug. The used concentrations of dexamethasone and cyproheptadine are 30.0 and 5.0 µg/mL for ultraviolet and fluorescence detection, respectively.

Difference in their elution order was revealed by the difference in lipophilicity between IND and the internal standard as indicated by Log P values which are 3.3, 1.93 and 5.027 for IND, dexamethasone and cyproheptadine, respectively as shown in fig.2 &3.

3.2 Method Validation

The developed analytical method was then subjected to method validation according to ICH Q2(R1) guidelines[8]. The following parameters were considered: linearity, sensitivity, LOD, LOQ, accuracy and precision.

3.2.1 Linearity

Under the above described experimental conditions, a linear relationship was established by plotting the peak area ratio [drug/I.S.] against the drug concentration in µg/mL. The concentration ranges were found to be 2.0-20 µg/mL and 0.05-5.0 µg/mL for ultraviolet and fluorescence detection, respectively (Table II).

Linear regression analysis of the data by the proposed method gave the following equations:

PA = -0.61 + 0.81 C (r =0.9999) ultraviolet detector

PA = 0.14 + 0.2 C (r =0.9999) fluorescence detector

Where, PA is the Peak area ratio, C is the concentration of the drug (µg/mL) and r is correlation coefficient. Statistical analysis of the data gave high value of the correlation coefficients (r) of the regression

equations, small values of the standard deviation of residuals ($S_{y/x}$), intercepts (S_a), and slopes (S_b), and small values of the percentage relative standard deviations and the percentage relative errors (Tables II). These data proved the linearity of the calibration graph.

3.2.2 Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ and LOD were calculated according to ICH Q2(R1) recommendations[8] using the following equations:

$$LOQ = 10\ S_a/b \text{ and } LOD = 3.3\ S_a/b$$

Where, S_a = standard deviation of the intercept and b = slope of the calibration curve

The limit of quantitation (LOQ) was determined by establishing the lowest concentration of the analyte that can be measured and below which the calibration graph is non linear. While, the limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected. Values of LOQ were 0.352 and 0.026 µg/mL, whereas, values of LOD were 0.116 and 0.009 µg/mL using UV and fluorescence detection, respectively.

3.2.3 Precision

The intra-day and inter-day precisions of the proposed HPLC method was examined by triplicate analysis of IND at three different concentrations 3.0, 9.0 and 12.0 µg/mL and 0.5, 1.0 and 2.0 µg/mL for ultraviolet and fluorescence detection, respectively at one day and for three consecutive days. The precision of the proposed method was satisfactory, as indicated by the low values of SD and RSD, also the low values of % Er indicates good accuracy of the method (Table III).

3.2.4. Accuracy

To prove the accuracy of the proposed method, the results of the assay of the studied drug in pure and dosage form were compared with those of the comparison method[5].

Statistical analysis[9] of the results obtained by the proposed and the comparison method using Student's t-test and variance ratio *F*-test revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Tables IV and V) ,since the calculated values did not exceed the tabulated ones.

3.2.5 Robustness

The steadiness of the peak area of IND with the intentional minor changes in the chromatographic conditions approve the robustness of the proposed method; these changes include; pH (3.0 ± 0.1), proportion of mobile phase (Buffer: ACN, 40: 60 v/v ± 2%), and flow rate (1.0 ± 0.1) mL/min for both detection modes. The peak area of IND drug was not highly influenced by these deliberate minor changes (Table VI).

3.2.6 Selectivity

The selectivity of the proposed method was proven by its ability to determine **IND** in its capsules without interference from the common excipients. The results were summarized in Table V.

3.2.7 System suitability test (SST)

Evaluation of SST parameters was performed during the development and optimization of the method (Table I). Moreover, to ascertain the effectiveness of the final operating system it was subjected to suitability testing. The test was performed by injecting the standard mixture in triplicate and the parameters were calculated as reported by the USP[6]. SST parameters include tailing factor *(T)* and column efficiency (number of theoretical plates, *N*).

3.3 Assay of pharmaceutical preparation

The proposed methods were applied successfully to the determination of IND in its capsules where no apparent interference from the capsule excipients (lactose) was noticed as illustrated by the placebo peaks. The potency of very low dose of Indacaterol (150 µg/mL) in its onbrez capsule combined by fast onset of action together with a dosing regimen compatible with once-daily dosing[10] .Standard addition method was applied to test the validity of the proposed methods. The recovery of IND, was determined by adding a known amount of pure drug at three different concentrations of 1.0, 3.0, and 6.0 µg/Ml for UV detector and 0.5, 1.0, and 2.0 µg/mL for fluorescence detector to previously analyzed capsule solution at two different concentrations 1.5 and 3.0 µg/mL for UV detector and 0.5, and 1.0 µg/mL for fluorescence detector. These concentrations of the pure drug were added in separate flasks to each capsule concentration and each solution was reanalyzed for the total drug content. The analysis was carried out in triplicate and was performed as described under “Construction of calibration curves”. The obtained results are shown in table VII. These control experiments eliminate the interference due to interactions of other constituents encountered in the system or caused by the bulk production.

4. Conclusion

The proposed method applies a validated HPLC to determine IND in pure form and in its capsule dosages form. The proposed method presents rapid (retention time is less than 5 min), sensitive (LOD values were 0.116 and 0.004 µg/mL adopting ultraviolet and fluorescence detection, respectively. Moreover, the proposed method was applied efficiently to analyze dosage form capsules in quality control laboratories.

REFERENCES

[1] O'Neil M. J. Royal Society of Chemistry. The Merke Index, 14th ed.; Whitehouse Station, New Jersey: Merke & co., Inc., Electronic version, 2013.

[2] Han, J., Dai, L.; Zhong, N. Indacaterol on dyspnea in chronic obstructive pulmonary disease: a systematic review and meta-analysis of randomized placebo-controlled trials. BMC pulm med 2013; 13 (26): 1-8.

[3] Emotte, C.; Heudi, O.; Deglave, F.; Bonvie, A.; Masson, L.; Picard, F.; Chaturvedi, A.; Majumdar, T.; Agarwal, A.; Woessner, R. et al. Validation of an on-line solid-phase extraction method coupled to liquid chromatography-tandem mass spectrometry detection for the determination of Indacaterol in human serum. J chromatogr B Analyt Technol Biomed Life Sci 2012; 895-896:1-9.

[4] Ammari, W.G.; Al-Qadhi, Z.; Khalil, M.; Tayyem, R.; Qammaz, S.; Oriquat, G.; Basheti, I.A.; Chrystyn, H. Indacaterol Determination in Human Urine: validation of a Liquid-Liquid Extraction and Liquid Chromatography-Tandem Mass Spectrometry Analytical Method. J Aerosol Med Pulm Drug Deliv, 2015; 28 (3):202-10.

[5] El-Ashry, S.M.; El-Wasseef, D.R.; El-Sherbiny, D.T.; Salem, Y.A. Spectrophotometric and spectrofluorimetric determination of indacaterol maleate in pure form and pharmaceutical preparations: application to content uniformity. J Luminescence, 2015. doi: 10.1002/bio.2838.

[6] United States Pharmacopoeial Convention. United States Pharmacopoeia, USP 34 NF 25 Inc, Rockville, MD, USA; Electronic Version, 2011: 905

[7] Clarke, E.G.C.; Moffat, A.C. Clarke's Analysis of Drugs and Poisons: In Pharmaceuticals, Body Fluids and Postmortem Material; London, Pharmaceutical Press, 2011.

[8] ICH Expert Working Group. ICH Harmonized Tripartite Guideline: validation of Analytical Procedures. Text and Methodology, Q2(R1), Current Step 4 Version, Parent Guidelines on Methodology Dated November 6 (1996), Incorporated in November 2005. http://www.ich.org/LOB/media/MEDIA417.pdf (accessed 15 February 2008).

[9] Miller, J. C.; Miller, J. N. Statistics and Chemometrics for Analytical Chemistry, 5th ed.; Harlow ,Pearson Education Limited, 2005.

[10] Trifilieff, A, Charlton, S, Fairhurst, R. The Preclinical Pharmacology of Indacaterol. In: Indacaterol. Edited by Trifilieff A. Springer Basel 2014; 25-37.

Tables

Table I. Optimization of the chromatographic conditions for the determination of IND by the proposed HPLC method using UV and fluorescence detection.

Parameter		Peak area		No. of theoretical plates (N)		Tailing factor	
		UV detector	Fluorescent detector	UV detector	Fluorescent detector	UV detector	Fluorescent detector
pH of Buffer	**3.0**	298.2	6200	1050.3	1710.8	1.05	1.08
	3.5	231.3	6064	520.7	1411.2	1.27	1.59
	4.0	155.2	6515	980.5	1449.3	1.27	1.6
	5.0	209.5	1295	900.9	1295.2	1.1	1.52
	6.0	232.4	5854	710.9	1143.8	2.0	1.45
Ratio of (Phosphate buffer : ACN (v/v))	**(30:70)**	Un-retained					
	(40:60)	245.2	1846	400.3	1575.1	1.25	1.32
	(50:50)	194.1	1931	450.9	1500.2	1.12	1.30
	(60:40)	298.2	6200	1050.5	1710.8	1.05	1.08
	(70:30)	110.9	145.2	310.69	1667.53	1.21	1.25
Effect of flow rate (mL/min)	**0.6**	187.9	4166	555.4	1758.8	1.62	1.44
	0.8	258.5	4108	550.02	1500.8	1.21	1.74
	1.0	298.4	6200	1050.3	1710.8	1.05	1.08
	1.2	179.3	4198	530.7	846.6	1.01	1.02

Where: Number of theoretical plates (N) = $5.45(t_R/ W_{h/2})^2$

- t_R is the retention time of the substance measured from the point of injection.
- Tailing factor (T) = $W_{h/2}/2f$.
- f = leading edge of the peak
- $W_{h/2}$ is the peak width at the half height

Table II. Performance data for the determination of IND using RP-HPLC adopting UV and fluorescence detection

Parameter	UV detectoion	Fluorescence detection
Concentration range (µg/mL)	2-20.0	0.05-5.0
Correlation coefficient	0.9999	0.9999
Slope	0.81	0.2
Intercept	-0.61	0.14
LOD (µg/mL)	0.116	8.6×10^{-3}
LOQ (µg/mL)	0.352	26.1×10^{-3}
$S_{y/x}$	0.041	1.0×10^{-3}
S_a	0.028	5.0×10^{-4}
S_b	0.003	6.0×10^{-4}
% RSD	0.476	0.471
% Er	0.168	0.166

N.B. -Sy/x =standard deviation of the residuals.

-Sa = standard deviation of the intercept of regression line.

-Sb = standard deviation of the slope of regression line.

-% Error = RSD% / √ n.

Table III. Accuracy and precision data for the determination of the IND by the proposed HPLC method.

		UV detection concentration (µg/mL)			Fluorescence detection concentration (µg/mL)		
		3.0	9.0	12.0	0.5	1.0	2.0
Intra-day	$\bar{x}$	100.76	99.31	100.08	99.52	100.44	100.22
	± SD	0.12	0.10	0.15	0.33	0.16	0.11
	% RSD	0.12	0.10	0.15	0.33	0.16	0.11
	% Er	0.07	0.06	0.09	0.19	0.09	0.07
Inter-day	$\bar{x}$	100.34	99.59	99.72	99.38	100.40	100.12
	± SD	0.53	0.28	0.13	0.22	0.13	0.24
	% RSD	0.53	0.28	0.13	0.23	0.13	0.24
	% Er	0.31	0.16	0.08	0.13	0.08	0.14
(*) Each result is the mean recovery of three separate determinations.							

Table (IV). Assay results for determination of IND in pure form by the proposed HPLC and comparison methods.

	Proposed RP-HPLC method				Comparison method[5]			
	Conc. taken (µg/mL)		% found		Conc. taken (µg/mL)		% found	
	UV detector	Fluorescent detector	UV detector	Fluorescent detector	Spectrophotometric method	Fluorimetric method	Spectrophotometric method	Fluorimetric method
Data	2.0	0.05	100.25	100.36	2.0	0.01	100.68	99.32
	3.0	0.08	100.79	99.04	6.0	0.02	99.02	99.90
	6.0	1.0	100.96	99.21	10.0	0.04	99.98	100.00
$\bar{X}$			100.7	99.54			99.89	99.74
± SD			0.37	0.72			0.83	0.37
t-value			1.4 (2.77)	0.43 (2.77)				
F-value			5.05 (19.0)	3.82 (19.0)				

Each result is the mean recovery of three separate determinations.

Figures between brackets are the tabulated *t* and *F*-values at (P= 0.05).

Table (V). Assay results for determination of IND in capsule dosage form by the proposed HPLC and comparison methods.

	Proposed RP-HPLC method				Comparison method[5]			
	Conc. taken * (µg/mL)		% found		Conc. taken (µg/mL)		% found	
	UV detector	Fluorescent detector	UV detector	Fluorescent detector	Spectrophotometric method	Fluorimetric method	Spectrophotometric method	Fluorimetric method
Data	2.0	1.0	100.35	100.39	1.0	0.003	100.77	99.32
	3.0	2.0	100.81	99.72	6.0	0.006	99.23	100.68
	6.0	3.0	100.63	100.10	9.0	0.009	100.26	99.77
$\bar{X}$			100.60	100.07			100.03	99.91
± SD			0.23	0.34			0.65	0.57
t-value			1.079 (2.77)	0.33 (2.77)				
F-value			11.45 (19.0)	4.25 (19.0)				

*****Each Onbrez capsule contains 150µg Ind. Mal., Product of Novartis & Chem.Ind. Co., batch no. 143984.

Each result is the mean recovery of three separate determinations.

Figures between brackets are the tabulated *t* and *F*-values at (P= 0.05).

Table VI. Robustness testing for the assay of Indacaterol maleate by the proposed HPLC method:

Content of Indacaterol maleate using ultraviolet and fluorescent detectors										
Parameter	**UV detection Sample number**					**Fluorescence detecion Sample number**				
	1	**2**	**3**	**Mean**	**%RSD**	**1**	**2**	**3**	**Mean**	**%RSD**
Flow Rate (mL/min)										
0.9	99.30	99.86	99.60	99.59	0.28	100.26	100.57	100.49	100.44	0.16
1.0	99.98	100.25	99.70	100.08	0.15	100.07	100.14	100.37	100.17	0.11
1.1	99.30	99.22	99.42	99.31	0.1	98.7	99.7	99.8	99.4	0.61
Buffer pH										
2.9	101.0	101.2	100.9	101.0	0.15	99.20	99.50	99.96	99.52	0.33
3.0	100.75	100.48	100.79	100.34	0.31	99.20	99.32	99.62	99.38	0.22
3.1	99.8	102.8	99.4	100.7	1.85	101.4	101.1	101.1	101.2	0.17
Mobile phase composition (acetonitrile: phosphate Buffer, V/V										
(42: 58, V/V)	100.63	100.87	100.79	100.76	0.12	100.26	100.42	100.52	100.44	0.16
(40: 60, V/V)	99.98	99.86	100.01	99.72	0.13	100.5	100.2	100.5	100.4	0.17
(38: 62, V/V)	101.2	100.2	99.9	100.4	0.68	99.8	102.8	99.4	100.7	1.85

Each result is the mean recovery of three separate determinations

Table VII. RP-HPLC method for determination of IND using UV and Fluorescence detection applying the standard addition method**:**

Method	**Capsule* concentration (µg/ mL)**	**Concentrationof added standard (µg/ mL)**	**Recovery± RSD (%)of added IND**	**Mean Recovery ± RSD (%) of added IND**
UV detector	1.5	1.0	100.35 ± 0.2	
	1.5	3.0	100.81 ± 0.08	100.51 ± 0.21
	1.5	6.0	100.37 ± 0.35	
	3.0	1.0	99.97 ± 0.76	
	3.0	3.0	100.63 ± 0.35	100.3 ± 0.44
	3.0	6.0	99.30 ± 0.2	
Fluorescence detector	0.5	0.5	100.39 ± 0.14	
	0.5	1.0	99.59 ± 0.11	100.02 ± 0.13
	0.5	2.0	100.09 ± 0.14	
	1.0	0.5	99.61 ± 0.28	
	1.0	1.0	99.72 ± 0.23	99.81 ± 0.20
	1.0	2.0	100.10 ± 0.10	

***** Each Onbrez capsule contains 150 µg Ind. Mal.,Product of Novartis &Chem.Ind. Co., batch no. 143984.

Each result is the mean recovery of three separate determinations

Figures

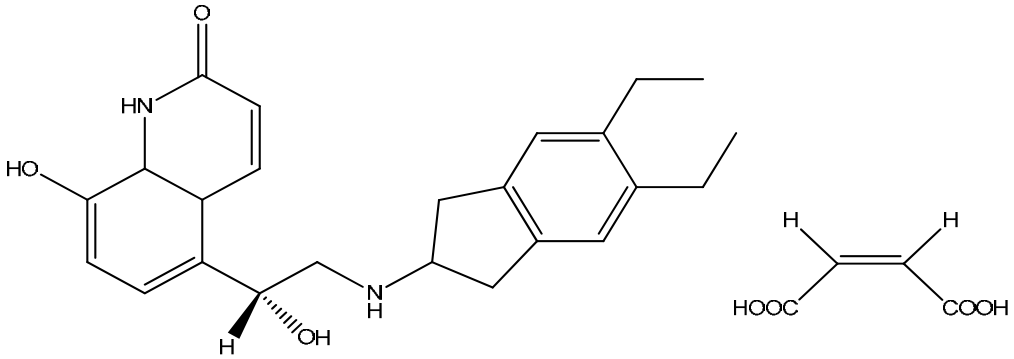

Fig. (1): Chemical structure of Indacaterol maleate (IND)

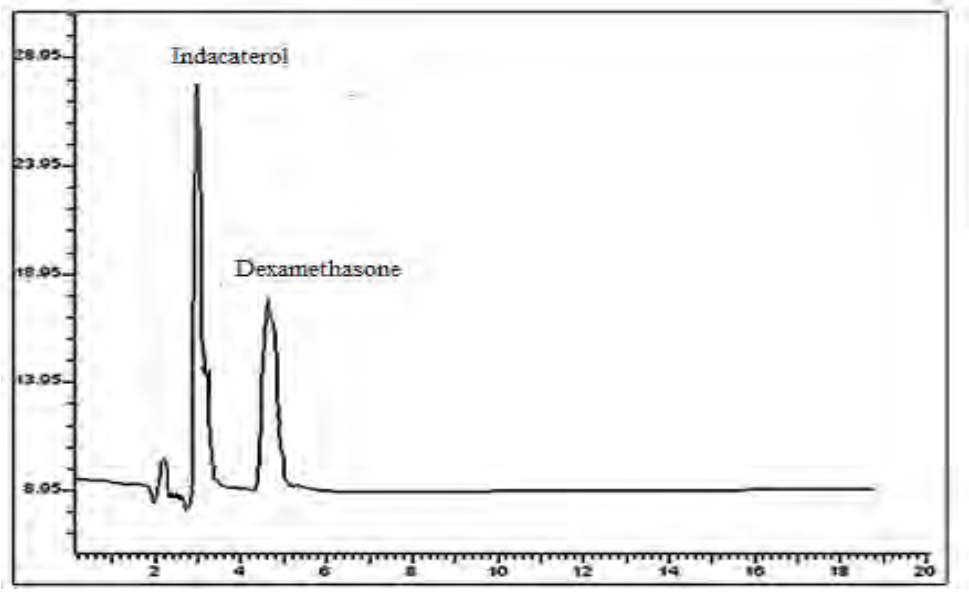

Fig.(2): Typical chromatogram for the separation of Indacaterol maleate (12.0 µg/mL, 3.0 min) and dexamethasone (30.0 µg /mL, 4.6 min) using Rp-HPLC with ultraviolet detection at 259 nm and C18, pre-packed column RT (150 mm × 4.6 mm ID, Lichrosorb® RP-18 (5 µm particle size). The mobile phase composition was acetonitrile: 0.05 M acid hydrogen phosphate buffer containing 0.3 % TEA (40: 60, %, V/V) adjusted to pH 3.0 using 0.02 M OPA.

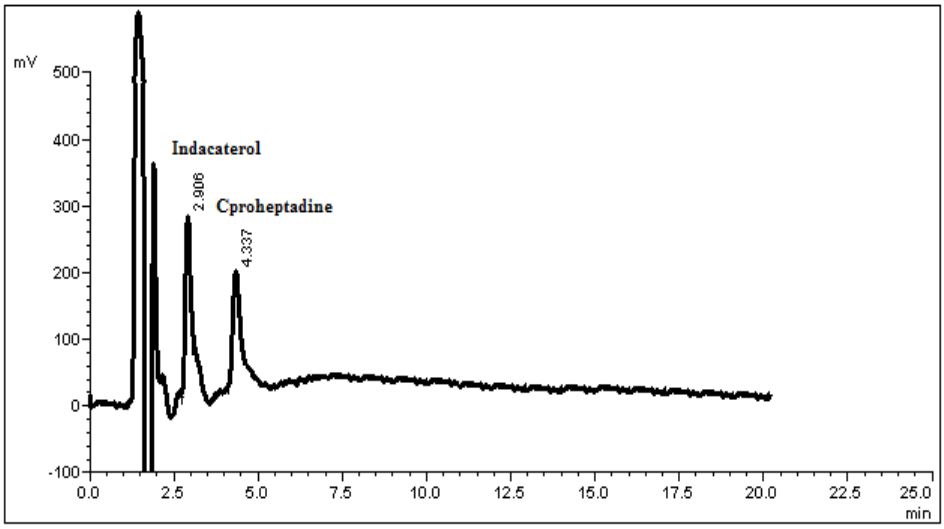

Fig. (3): Typical chromatogram for the separation of Indacaterol maleate (5.0 µg/mL, 2.9 min) and cyproheptadine (5.0 µg /mL, 4.3 min) using Rp-HPLC with fluorescence detection (λex/em: 258/421 nm) and C18, pre-packed column RT. The mobile phase composition was acetonitrile: 0.05 M acid hydrogen phosphate buffer containing 0.3 % TEA (40: 60 %, V/V) adjusted to pH 3.0 using 0.02 M OPA.