WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 7, Issue 3, 166-176 Research Article ISSN 2278 – 4357

RP-HPLC-DAD METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF INDACATEROL AND GLYCOPYRRONIUM IN THEIR PHARMACEUTICAL FORMULATION

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Article Received on
05 Jan. 2018,
Revised on 26 Jan. 2018,
Accepted on 17 Feb. 2018
DOI: 10.20959/wjpps20183-11151

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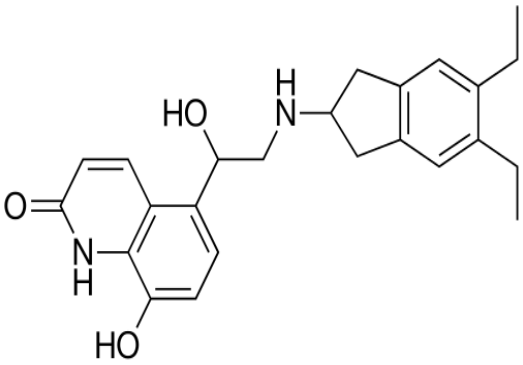
ABSTRACT

A simple, sensitive and accurate reversed phase HPLC method was developed and validated for the simultaneous determination of indacaterol maleate and glycopyrronium bromide in commercial powder for inhalation capsules. The method has shown adequate separation of indacaterol maleate and glycopyrronium bromide. Separation was achieved on supelco C18 column (25 cm X 4.6mm, 5µm particle size) using mobile phase consists of acetonitrile: methanol: water (50:30:20, by volume). The retention times for glycopyrronium bromide and indacaterol maleate were 4.051 and 5.310 minutes respectively. Linearity of the proposed method was investigated in the range of 1-12 µg/ml (r^2 =0.9998) for indacaterol maleate and 0.5-6 µg/ml (r^2 =0.9998) for glycopyrronium bromide. The limit of detection (LOD) was 0.1749 µg/ml for indacaterol maleate and 0.0341µg/ml for glycopyrronium bromide. The limit of quantification (LOQ) was 0.5301 µg/ml for indacaterol maleate and 0.1034 µg/ml for glycopyrronium bromide. The proposed method was validated according to International Conference on Harmonization (ICH) guidelines, statistically compared with a reported spectrophotometric method and successfully applied for the simultaneous determination of both drugs in their combined dosage form.

KEYWORDS: Indacaterol maleate, Glycopyrronium bromide, RP-HPLC-DAD.

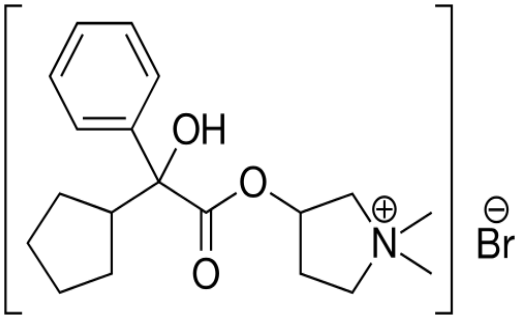
INTRODUCTION

Indacaterol maleate is 5-{(1*R*)-2-[(5,6-diethyl-2,3-dihydro-1*H*-inden-2-yl)amino]-1-hydroxyethyl}-8-hydroxy-2(1*H*)-quinolinone maleate (Fig.1). It's white to very slightly grayish or very slightly yellowish not hygroscopic powder. It is soluble in methanol , slightly soluble in water, insoluble in 0.9% NaCl.[1] Glycopyrronium bromide is 3-(2-cyclopentyl-2-hydroxy-2-phenylacetoxy)-1,1- dimethylpyrrolidinium bromide (Fig.2). It's a white, non-hygroscopic powder, freely soluble in water, soluble in methanol (96%), very slightly soluble in methylene chloride.[1] Ultibro® Breezhaler is a combination of indacaterol maleate and glycopyrronium bromide licensed as a maintenance bronchodilator treatment to treat chronic obstructive pulmonary disease in which indacaterol maleate is long acting β_2 agonist and glycopyrronium bromide is long acting antimuscarinic.[2] There is only one reported spectrophotometric method for simultaneous determination of indacaterol maleate and glycopyrronium bromide.[3] Few LC-MS methods were reported for determination of indacaterol maleate in plasma and urine.[4-6] Spectrophotometric method were also reported for determination of indacaterol maleate in pharmaceutical formulation[7], Literature survey revealed that different methods for determination of glycopyrronium bromide in biological fluids using LC-MS[8,9], HPLC[10], GC[11], visible spectrophotometric method.[12] The objective of the present work is the new development and validation of a RP-HPLC method for the estimation of indacaterol maleate and glycopyrronium bromide in bulk and inhalation capsules.



M. wt.: 508.57
M. formula: $C_{28}H_{32}N_2O_7$

Figure. (1): Structural formula of indacaterol maleate.



M. wt. : 398.34
M. formula: $C_{19}H_{28}BrNO_3$

Figure. (2): Structural formula of glycopyrronium bromide.

Experimental

Instruments

- HPLC, constaMetric® 4100 LDC analytical pump (Milton Roy, USA), equipped with Spectra system UV3000 diode-array UV-Visible detector and spectra system AS3000 auto sampler. The chromatographic analysis was carried out using (ChromQuest 4.2.34, version 3.1.6) data analysis program.

Materials

Pure samples

- Pure indacaterol maleate (99.7 %) was kindly supplied by Novartis Company, Egypt.
- Pure glycopyrronium bromide (99.5 %) was kindly supplied by Novartis Company, Egypt.

Pharmaceutical preparation:

- Ultibro® Breezhaler: each capsule claimed to contain 143µg indacaterol maleate and 63 µg glycopyrronium bromide (B.No.S0308, manufactured by Novartis pharma stein, Switzerland), purchased from local market.

Chemicals and solvents

- Acetonitrile HPLC grade (Sigma-Aldrich, Germany).
- Methanol HPLC grade (Sigma-Aldrich, Germany).
- Distilled water.

General procedure

Chromatographic conditions: At ambient temperature, isocratic separation was carried out on supelco C18 column (25 cm X 4.6mm , 5µm particle size) using mobile phase consists of

acetonitrile: methanol: water (50:30:20, by volume). The mobile phase was degassed by a degasser before pumped at flow rate of 1ml/min.The injected volume of the standard solution was 20 µl and UV detection at 220 nm.

Preparation of standard solutions: A stock standard solution of indacaterol maleate (100 µg/ml) was prepared by dissolving 10 mg of the drug powder in 50 ml of the mobile phase, acetonitrile: methanol: water (50:30:20, by volume), and the volume was completed to 100 ml with the mobile phase. A stock standard solution of glycopyrronium bromide (100µg/ml) was prepared by dissolving 10 mg of the drug powder in 50 ml of the mobile phase and the volume was complete to 100 ml with the mobile phase.

Construction of the calibration graph: Aliquots of standard indacaterol maleate solution (100µg/ml) equivalent to (10–120µg) and glycopyrronium bromide (10µg/ml) equivalent to (5–60µg) were transferred into two separate series of 10-ml volumetric flasks and adjusted to volume with the mobile phase. Into HPLC column, 20 µl were injected from each solution and eluted with the mobile phase under the previously described chromatographic conditions.

Procedure for pharmaceutical preparation

Contents of ten **Ultibro®Breezhaler** capsules were mixed and weighed. All amount equivalent to ten capsules content (1430 µg of indacaterol maleate and 630 µg of glycopyrronium bromide) was extracted by shaking with 50 ml of mobile phase for 15 minutes, then filtered into 100 ml volumetric flask and the volume was adjusted with the mobile phase to obtain a solution labeled to contain (14.3 µg/ml of indacaterol maleate and 6.3 µg/ml glycopyrronium bromide).Apply the general procedure using aliquots covering the working concentration range. Determine the content of the Breezhaler® capsules from the corresponding regression equation.

RESULTS AND DISCUSSIONS

In the present study, very sensitive reversed phase HPLC procedure was suggested for the simultaneous quantitative determination of binary mixture of indacaterol maleate and glycopyrronium bromide.

Method Development: Different chromatographic conditions affecting the chromatographic separation were optimized after taking in consideration the achievement of sufficient resolution between both drugs. Several mobile phases including (acetonitrile: methanol:

water) in different ratios were tried in order to separate indacaterol maleate and glycopyrronium bromide. Good separation was carried out on supelco C18 column (25 cm X 4.6mm, 5µm particle size) using a mobile phase consists of acetonitrile: methanol: water (50:30:20, by volume) at flow rate 1ml/min and UV detection at 220 nm.

HPLC chromatogram revealed that glycopyrronium bromide and indacaterol maleate were clearly separated from each other at retention times of 4.051 + 0.032 and 5.310 + 0.052 minutes respectively, as shown in **(Fig.3).**

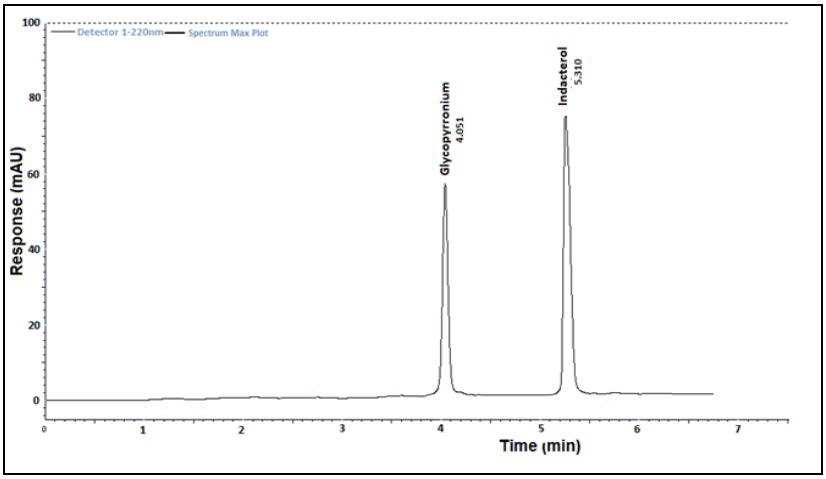


Figure. (3): HPLC chromatogram of mixture of indacaterol maleate (1µg/ml), and glycopyrronium bromide (0.5µg/ml).

Method Validation[14]

The proposed RP-HPLC method was validated according to International Conference on Harmonization (ICH) guidelines.

(a) Linearity and range: The linearity of the developed method was determined and the regression data for the calibration curve showed good linearity for both drugs (r^2 = 0.9998) for indacaterol maleate and (r^2 = 0.9998) for glycopyrronium bromide. The regression plots were found to be linear over the ranges of 1-12 µg/ml for indacaterol maleate and 0.5-6 µg/ml for glycopyrronium bromide. The linear regression equations for the graphs are

- **For indacaterol maleate**

y **= 10486.62** x **-330.53** **(r^2 = 0.9998).**

- **For glycopyrronium bromide**

y **= 8164.22** x **+58.26** **(r^2 = 0.9998).**

Where **y** is the peak area, **x** is the drug concentration in µg/ml and $\mathbf{r^2}$ is coefficient of determination.

Linearity ranges, regression equations, intercepts, slopes and coefficients of determination were presented in **table 1.**

(b) Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated from the following equations:

LOD = 3.3 σ / S

LOQ = 10 σ / S

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve. LOD and LOQ values of glycopyrronium and indacaterol for the proposed method were listed in **table (1)**.

Table. 1: Validation sheet and regression parameters of indacaterol maleate and glycopyrronium bromide by the proposed HPLC method.

Parameters		**Proposed HPLC method**	
Drug		**Indacaterol**	**Glycopyrronium**
Wavelength (nm)		220	
Linearity range (µg/ml)		1 ─ 12	0.5-6
- Regression Equation - Slope (b) - Intercept (a)		$y = b\,x + a$ 10486.6 -330.5	$y = b\,x + a$ 0.8164.2 58.2
Coefficient of determination (r^2)		0.9998	0.9998
Accuracy (%R)*		99.73	99.88
Precision (%RSD)	Repeatability	0.565	0.364
	Intermediate precision	0.585	0.564
LOD (µg/mL)		0.1749	0.0341
LOQ (µg/mL)		0.5301	0.1034

*****Average of three determination.

(c) Accuracy

The accuracy of the method was calculated by measuring the mean percent recovery of three determinations for three concentration of the studied drugs.

(d) Precision: The precision of the developed method was checked by measuring percent relative standard deviation (% RSD) of both drugs for three concentrations in the same day (repeatability) and in three days (intermediate precision). The obtained values confirming high precision of the method.

(e) Specificity

The specificity test of the proposed method demonstrated that the method was found to be selective to the drug due to well-shaped resolution found for the drug peak with no interference. Moreover the standard addition technique was applied to check the specificity of the described method. It was done by adding known quantities of glycopyrronium and indacaterol in its pure form to already analyzed pharmaceutical preparation and the percent recovery of the pure added concentrations was calculated. The data listed in **Table (2)** proved that the proposed method could selectively analyze the drug s without any interference from any excipients.

Table. 2: Standard addition technique of the proposed HPLC method.

Ultibro® Breezhaler capsules				
Indacaterol				
Pharmaceutical Taken (µg/ml)	**Pharmaceutical Found* (µg/ml)**	**Pure added (µg/ml)**	**Pure found (µg/ml)**	**Recovery %**
1.43	1.43	1	0.99	99.01
		1.5	1.47	98.20
		2	1.99	99.50
Mean				98.90
%RSD				0.662
Glycopyrronium				
Pharmaceutical Taken (µg/ml)	**Pharmaceutical Found* (µg/ml)**	**Pure added (µg/ml)**	**Pure found (µg/ml)**	**Recovery %**
0.63	0.63	1	0.99	99.44
		1.5	1.47	98.29
		2	1.97	98.39
Mean				98.71
%RSD				0.645

*Average of five determinations.

(f) System suitability tests

To confirm that, the chromatographic system was working correctly during the analysis process, various parameters such as the number of theoretical plates (N), resolution factor (Rs), retention factor ($\bar{k}$) and tailing factor (T) were evaluated. The results of l system suitability test parameters for the method were presented in **table (3)**.

Table. 3: System suitability results for the determination of indacaterol maleate and glycopyrronium bromide by the proposed HPLC method.

Parameters	Glycopyrronium	Indacaterol	Reference value[13]
Retention time (t_R)	4.051± 0.032	5.310± 0.052	---
Retention factor (K´)	1.39	2.34	1-10
Theoretical Plates (N)	2228	2206	>2000
Tailing factor (T)	0.786	0.658	< 2
Injection precision (%RSD)	0.463	0.632	< 1
Resolution (Rs)*	4.39		>2

*Related to indacaterol.

(g) Robustness

To measure the ability of the proposed method to remain unaffected by small, but deliberate variations of the method parameters, evaluation has been done by slight changes in the chromatographic conditions such as flow rate (±0.1 mL/min), and mobile phase contents ratio (±2%). In each case only one parameter was changed while other conditions were kept constant. The described minor changes did not affect the separation and resolution of glycopyrronium and indacaterol confirming the robustness of the proposed method. The results were given in **table (4).**

Table. (4): Robustness results for the determination of glycopyrronium and indacaterol by the proposed HPLC procedure.

Parameters		Retention time (t_R)	Tailing factor (T)	Resolution (Rs)*
Glycopyrronium				
Flow rate (ml/min.)	0.9	4.032	0.776	4.37
	1	4.051	0.786	4.39
	1.1	4.097	0.798	4.41
Mobile phase (acetonitrile: methanol:water)	52:29:19	4.033	0.772	4.36
	50:30:20	4.051	0.786	4.39
	48:31:21	4.089	0.799	4.43
Indacaterol				
Flow rate (ml/min.)	0.9	5.306	0.649	4.38
	1	5.310	0.658	4.39
	1.1	5.315	0.665	4.40
Mobile phase (acetonitrile: methanol:water)	52:29:19			4.36
	50:30:20	5.310	0.658	4.39
	48:31:21			4.41

***resolution related to indacaterol.**

Application to pharmaceutical formulations

The proposed procedure was applied for the simultaneous determination of indacaterol maleate and glycopyrronium bromide in **Ultibro®Breezhaler** inhalation capsules. Satisfactory results were obtained in good agreement with the label claim. The obtained results were statistically compared to those obtained by the reported method.[3] No significant differences were found by applying *t*-test and *F*-test at 95% confidence level,[15] indicating good accuracy and precision of the proposed methods for the analysis of the studied drug in its pharmaceutical dosage form, as shown in **table 5.**

Table. 5: Determination of indacaterol maleate and glycopyrronium bromide in Ultibro® Breezhaler capsules by the proposed HPLC and reported methods.

Parameters	Proposed method		Reported method[3]	
	Indacaterol	Glycopyrronium	Indacaterol	Glycopyrronium
Number of measurements	5	5	5	5
Mean	100.04	99.96	99.23	99.92
Variance	0.754	0.402	0.440	1.141
%RSD	0.868	0.634	0.668	1.069
Student's *t*-test	1.66 (2.36)*	0.07 (2.36)*	——	——
F-value	1.72 (6.39)*	2.84 (6.39)*	——	——

*The values in parenthesis are tabulated values of "*t* "and "*F*" at (P = 0.05).[3]

Spectrophotometric determination using savitzky golay filter, the peak amplitude at 264 and 236 nm corresponding to the concentrations of indacaterol and glycopyrronium.

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