iJRASET
International Journal For Research in Applied Science and Engineering Technology



INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 10 **Issue:** VI **Month of publication:** June 2022

DOI: https://doi.org/10.22214/ijraset.2022.44995

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 7.538
Volume 10 Issue VI June 2022- Available at www.ijraset.com

Formulation and Evaluation of Analytical Methods for Determination of Indacaterol

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Abstract: Indacaterol is a novel class of β2 adrenoreceptor -agonist used to treat chronic obstructive pulmonary disease. Various analytical methods used for the estimation of Indacaterol have been reviewed in this project. These methods include Ultraviolet Spectrometric method, High Performance Liquid Chromatography method, Capillary electrophoretic method, TLC densitometric method for qualitative and quantitative estimation of Indacaterol in pharmaceutical formulation

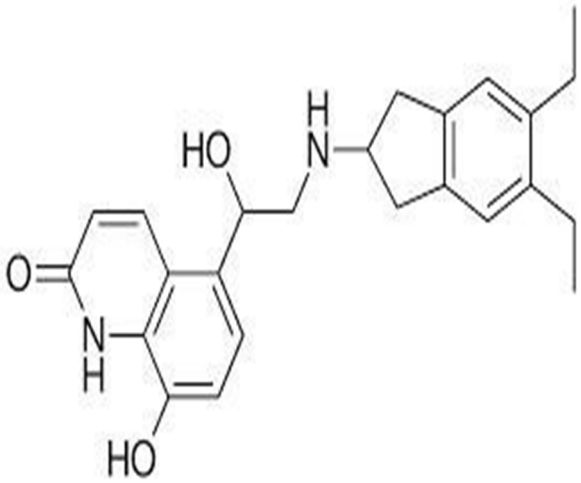
I. OBJECTIVE

The objective of the review study is qualitative and quantitative estimation of Indacaterol in bulk drug and pharmaceutical formulation by different anaytical methods such as UV Spectroscopic method, High Performance Liquid Chromatography method, Capillary electrophoretic method, TLC densitometric method.

II. INTRODUCTION

Indacaterol is in a class of medications called long-acting beta agonists (LABAs). It works by relaxing and opening air passages in the lungs, making it easier to breathe.

Structure: Indacaterol



Chemical name : 5-(2-(5,6-diethylindan-2-ylamino)-1-hydroxyethyl)-8-hydroxy-1Hquinolin2-one

Chemical Formula : C24H28N2O3

Molecular weight : 392.4907

Mechanism of Action:

Indacaterol works by stimulating adrenergic beta-2 receptors in the smooth muscle of the airways. This causes relaxation of the muscle, thereby increasing the diameter of the airways, which become constricted in asthma and COPD. It is also long acting due to its high affinity to the lipid raft domains in the airway membrane so it slowly dissociates from the receptors. Indacaterol also has a high intrinsic efficacy so it is also very rapid acting - onset of action occurs within 5 minutes.

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III. ANALYTICAL TECHNIQUES FOR ESTIMATION OF INDACATEROL:

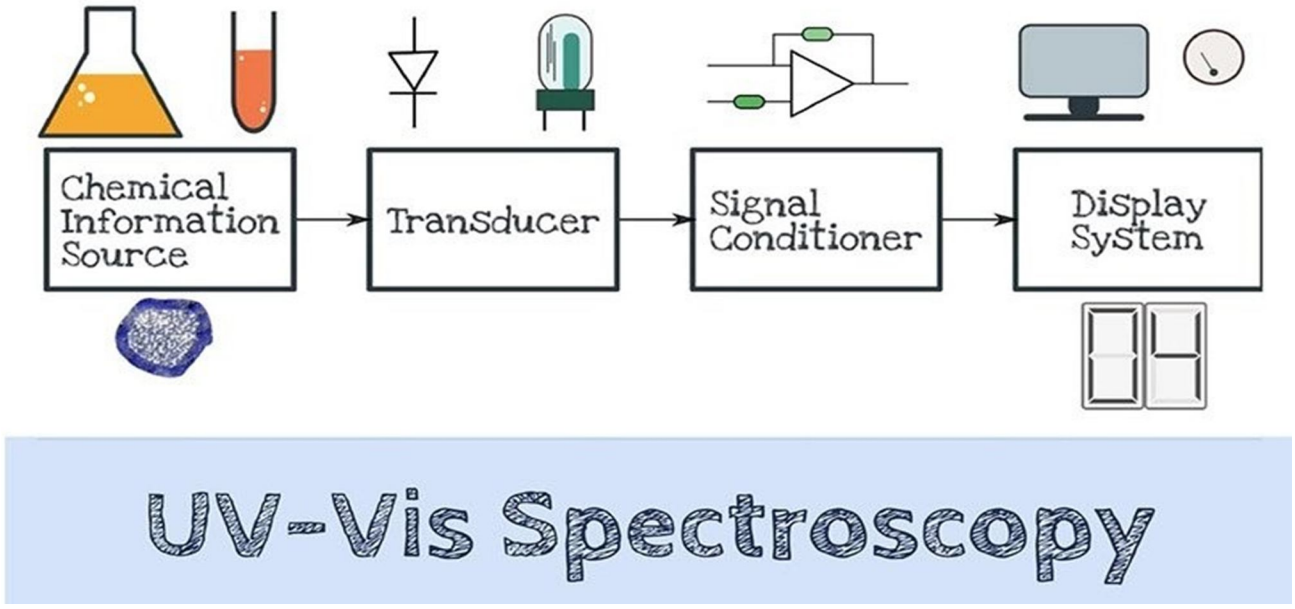
1) UV Spectroscopic method
2) IR Spectroscopy method
3) TLC Densitometric method
4) High Performance Liquid Chromatography method
5) Capillary electrophoretic method

A. UV Spectroscopic Method

Y.A. Salem et al.[3] developed Spectroscopic method for estimation of Indacaterol in capsules. Three simple and sensitive spectrophotometric methods (A, B and C) have been developed for the quantitative estimation of Indacaterol (IND) in bulk drug and capsules.

1) Principle

- Basically, spectroscopy is related to the interaction of light with matter.
- As light is absorbed by matter, the result is an increase in the energy content of the atoms or molecules.
- When ultraviolet radiations are absorbed, this results in the excitation of the electrons from the ground state towards a higher energy state.
- Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet light to excite these electrons to higher anti-bonding molecular orbitals.
- The more easily excited the electrons, the longer the wavelength of light it can absorb. There are four possible types of transitions (π–π*, n–π*, σ–σ*, and n–σ*), and they can be ordered as follows: σ–σ* > n–σ* > π–π* > n–π*
- The absorption of ultraviolet light by a chemical compound will produce a distinct spectrum which aids in the identification of the compound.



2) Apparatus

- Spectrophotometric analyses were carried out on a Shimadzu (Kyoto, Japan) UV-1601 PC, UV-Visible double-beam spectrophotometer with matched 1 cm path-length quartz cells (method A)
- Spectrophotometric analyses were carried out on JENWAY UV/ Visible Scanning Spectrophotometers 6315a United Kingdom with matched 1 cm path-length quartz cells.
  Absorption spectrum of the reaction product wasrecorded on a fast scan speed between 400710 nm setting slit width to be 1 nm and sampling interval to be auto (method B and C).

3) General Procedure

Preparation of stock and standard solutions

A stock solution (100 µg mL^{-1}) was prepared by dissolving 10.0 mg of Indacaterol (IND) in 100 mL methanol. Serial dilutions with the same solvent were prepared to obtain the appropriate concentration range. The standard solutions were stable for at least two weeks when kept in the refrigerator.

Construction of the Calibration Curves: (Method A)

Accurately measured aliquots equivalent to 1.0-16.0 µg IND was quantitatively transferred from the stock solution into two separate sets of 10 mL volumetric flasks. The set of the drug consists of two series. The first series of flasks were made up to the volume with 0.1 N NaOH and the absorption spectra were recorded against blank solution of 0.1 N NaOH at 274.6 nm. The flasks of the second series were made up to the volume with 0.1 N HCl and the absorption spectra were recorded against blank solution of 0.1 N HCl at 257.8 nm. The absorbance difference (δ A) between acidic solution and equimolar basic solution was measured by subtracting the spectra of the second series (in 0.1 N HCl) from the spectra of the first one (in 0.1 N NaOH) for each concentration of IND drug and the absorbance was taken immediately. The (δA) values of the difference absorption spectra were plotted vs. concentration of IND (µg mL^{-1}) to get the calibration graphs. Alternatively, the corresponding regression equation was derived.

Construction of the Calibration Curves (Method B)

Accurately measured aliquots of the stock solution were transferred into a series of 10-mL volumetric flasks to obtain the final concentration is in the range of 2.0 – 20.0 µg mL^{-1}. 1 mL of MBTH solution and 2.5 mL of ceric (IV) ammonium sulphate were added to each flask. The reaction mixture was mixed well and allowed to stand for 25 minutes at room temperature, then the flasks were made up to the volume with acetonitrile. The absorbance of the solution was measured at 545 nm against a reagent blank. Simultaneously, the calibration curve was constructed by plotting the values of the absorption spectra vs. concentration of IND (µg mL^{-1}). Alternatively, the corresponding regression equation was derived.

Construction of the Calibration Curves (Method C)

Aliquot volumes of IND standard solution were transferred into a series of 10- mL volumetric flasks to get final concentration within the range of 3.0- 30.0 µg mL^{-1}. Then 0.6 mL of ammonia solution, 2.0 mL of 4- aminoantipyrine solution, and 1.0 mL of potassium hexacyano ferrate solution were added to each flask. The reaction mixture was mixed well and allowed to stand for 15 minutes at thermostatic waterbath adjusted to 70°C then diluted to volume with distilled water. The absorbance values at 510 nm were measured against a reagent blank and then plotted against the final concentration to get the calibration graph. Alternatively, the corresponding regression equation was derived.

4) Results and Discussion

Method A was based on the measurement of the difference absorption spectra of Indacaterol in 0.1 N HCl and 0.1 N NaOH media. The difference spectrum exhibits maxima and minima at 274.6 and 257.8 nm, respectively. The method was found to be linear in the concentration range of 1 to 16 µg/ ml with percent recovery of 99.37% - 100.19%. The LOD and LOQ were found 0.07 µg /ml and 0.212 µg /ml respectively.

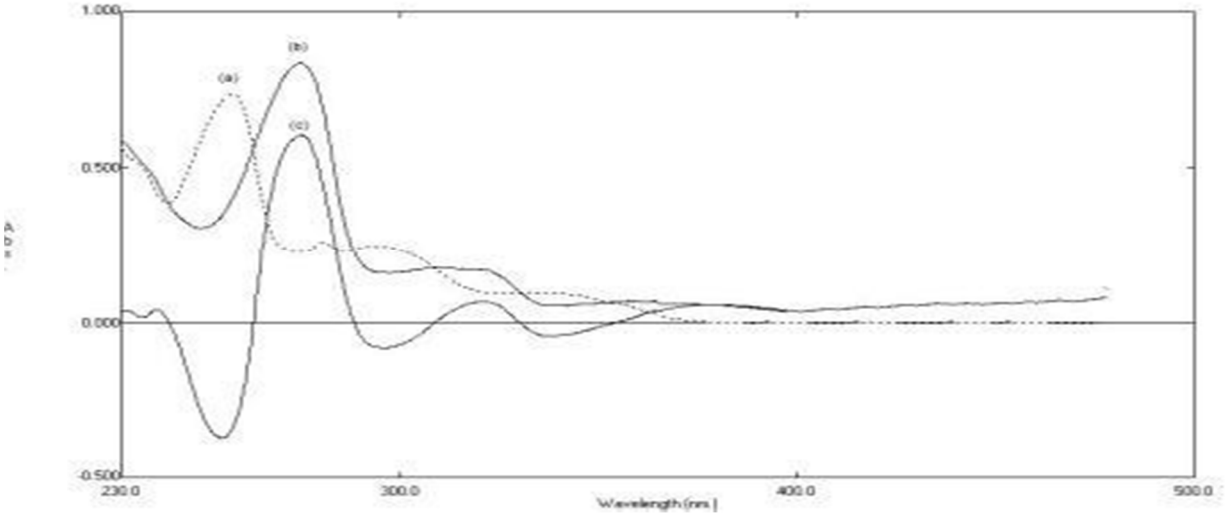


Fig: Absorption spectra of IND (12.0 µg mL^{-1}) in 0.1 N HCl, 0.1 N NaOH and difference absorption spectra of IND.

Method B was based on the oxidative coupling reaction with an acidic solution of the chromogenic agent 3-methylbenzothiazoline-2-one hydrazone (MBTH) and the drug upon treatment with ceric ammonium sulphate (CAS) produces an orange colour peaking at 545 nm. The method was found to be linear in the concentration range of 2 to 20 µg/ ml with percent recovery of 99.51% - 99.90%. The LOD and LOQ were found 0.213 µg /ml and 0.651 µg /ml respectively.

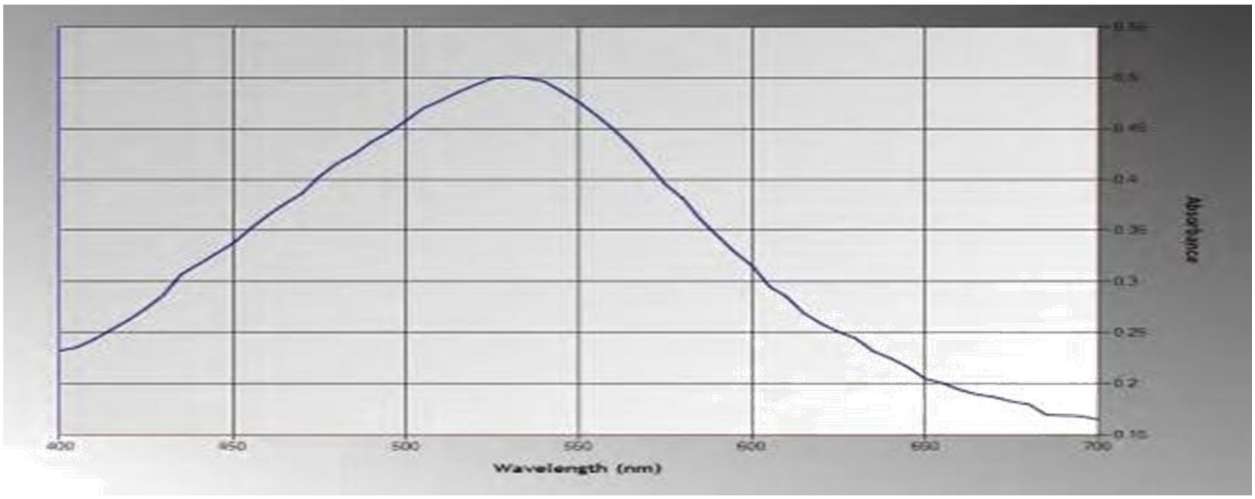


Fig: Absorption spectrum of the reaction product of Indacaterol (12.0 µg mL^{-1}) with MBTH-ceric ammonium sulphate system.

Method C was based on the reaction with 4-aminoantipyrine (4-AAP) in presence of alkaline oxidizing agent; potassium hexacyano ferrate and diluted ammonia (K3 [Fe (CN) 6] / NH3) and measuring the produced red colour at 510 nm. Beer's law is obeyed in the concentration range of 3.0 – 30.0 µg /ml with percent recovery of 99.60 - 100.54 %. The LOD and LOQ were found 0.125 µg /ml and 0.38 µg /ml respectively.

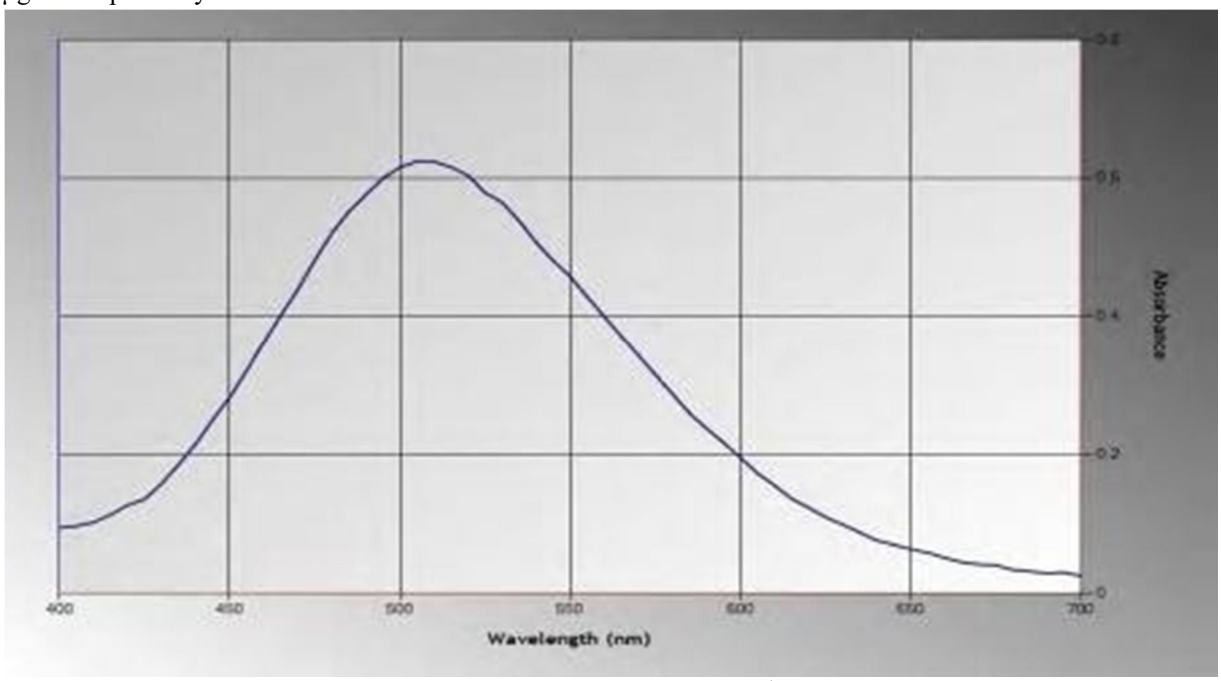


Fig: Absorption spectrum of the reaction product of Indacaterol (18.0 µg mL^{-1}) with 4aminoantipyrene (4-AAP). Conclusion:

Method A was found to be the most sensitive method providing the highest molar absorptivity and specific absorbance values. For application in quality control laboratories, method A is considered superior to other methods owing to its rapidness, minimum steps, simplicity and sensitivity providing high rates of sample throughput.
The proposed methods were found to be useful for routine analysis of pharmaceutical formulation.

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B. Infrared Spectroscopy

Infrared (IR) spectroscopy is one of the most common and widely used spectroscopic techniques employed mainly by inorganic and organic chemists due to its usefulness in determining structures of compounds and identifying them. Chemical properties due to the presence of different functional groups.

IR spectroscopy (which is short for infrared spectroscopy) deals with the infrared region of the electromagnetic spectrum, i.e. light having a longer wavelength and a lower frequency than visible light. Infrared Spectroscopy generally refers to the analysis of the interaction of a molecule with infrared light.

The IR spectroscopy concept can generally be analyzed in three ways: by measuring reflection, emission, and absorption. The major use of infrared spectroscopy is to determine the functional groups of molecules, relevant to both organic and inorganic chemistry.

1) What is IR Spectroscopy?

An IR spectrum is essentially a graph plotted with the infrared light absorbed on the Y-axis against. Frequency or wavelength on the X-axis. An illustration highlighting the different regions that light can be classified into is given below.

IR Spectroscopy detects frequencies of infrared light that are absorbed by a molecule. Molecules tend to absorb these specific frequencies fo light since they correspond to the frequency of the vibration of bonds in the molecule.

The energy required to excite the bonds belonging to a molecule, and to make them vibrate with more amplitude, occurs in the infrared region. A bond will only interact with the electromagnetic infrared radiation, however, if it is polar.

The presence of separate areas of partial positive and negative charge in a molecule allows the electric field component of the electromagnetic wave to excite the vibrational energy of the molecule. The change in the vibrational energy leads to another corresponding change in the dipole moment of the given molecule. The intensity of the absorption depends on the polarity of the bond. Symmetrical non-polar bonds in N=N and O=O do not absorb radiation, as they cannot interact with an electric field.

2) Samples in Infrared Spectroscopy

The samples used in IR spectroscopy can be either in the solid, liquid, or gaseous state.

- Solid samples can be prepared by crushing the sample with a mulling agent which has an oily texture. A thin layer of this mull can now be applied on a salt plate to be measured.
- Liquid samples are generally kept between two salt plates and measured since the plates are transparent to IR light. Salt plates can be made up of sodium chloride, calcium fluoride, or even potassium bromide.
- Since the concentration of gaseous samples can be in parts per million, the sample cell must have a relatively long pathlength, i.e. light must travel for a relatively long distance in the sample cell.

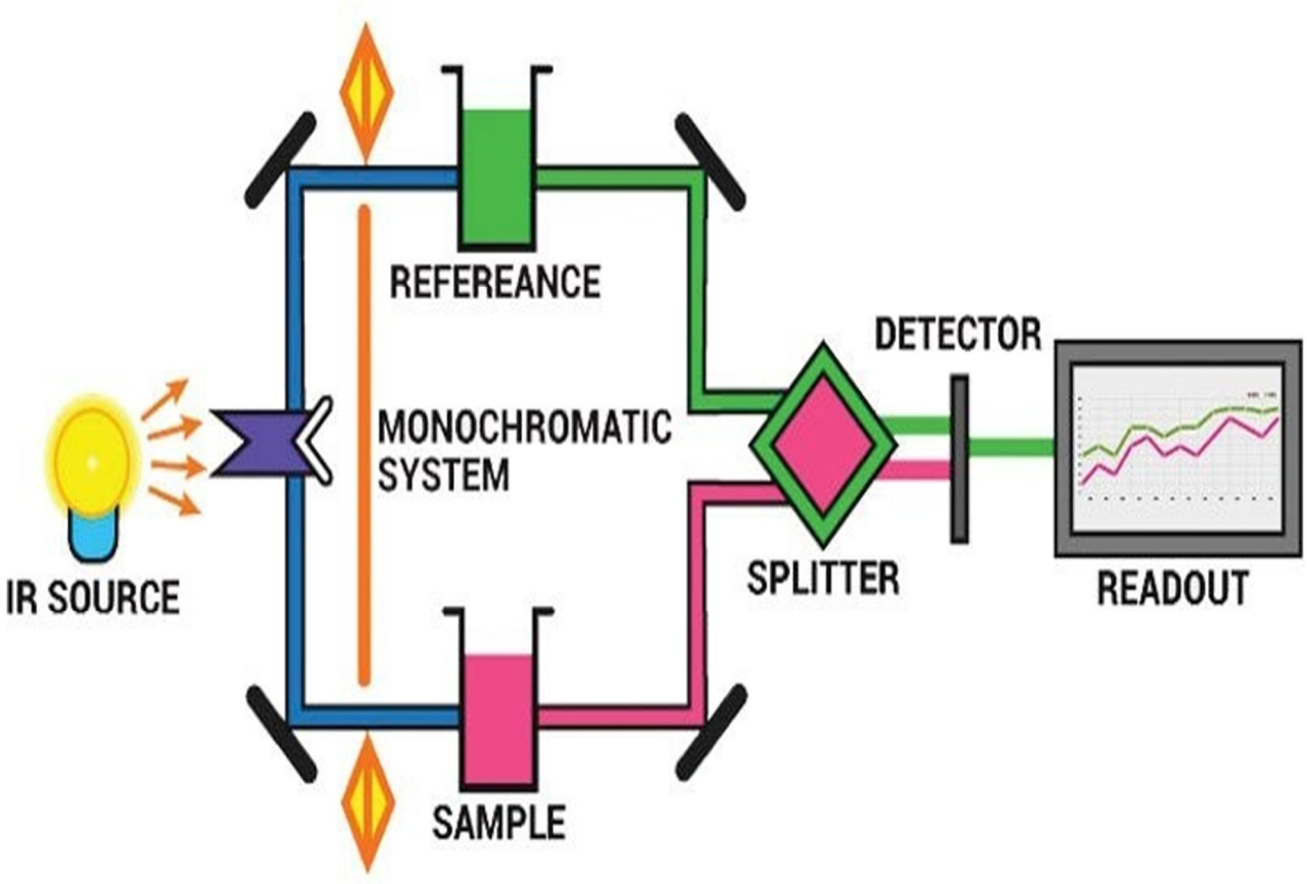
Thus, samples of multiple physical states can be used in Infrared Spectroscopy.

3) Principle Of Infrared Spectroscopy

- Infrared Spectroscopy is the analysis of infrared light interacting with a molecule.
- The portion of the infrared region most useful for analysis of organic compounds have a wavelength range from 2,500 to 16,000 nm, with a corresponding frequency range from 1.9*1013 to 1.2*1014 Hz.
- Photon energies associated with this part of the infrared (from 1 to 15 kcal/mole) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups.
- It is known that in addition to the facile rotation of groups about single bonds, molecules experience a wide variety of vibrational motions, characteristic of their component atoms.
- Consequently, virtually all organic compounds will absorb infrared radiation that corresponds in energy to these vibrations.
- Infrared spectrometers, similar in principle to other spectrometer, permit chemists to obtain absorption spectra of compounds that are a unique reflection of their molecular structure.
- The fundamental measurement obtained in infrared spectroscopy is an infrared spectrum, which is a plot of measured infrared intensity versus wavelength (or frequency) of light.

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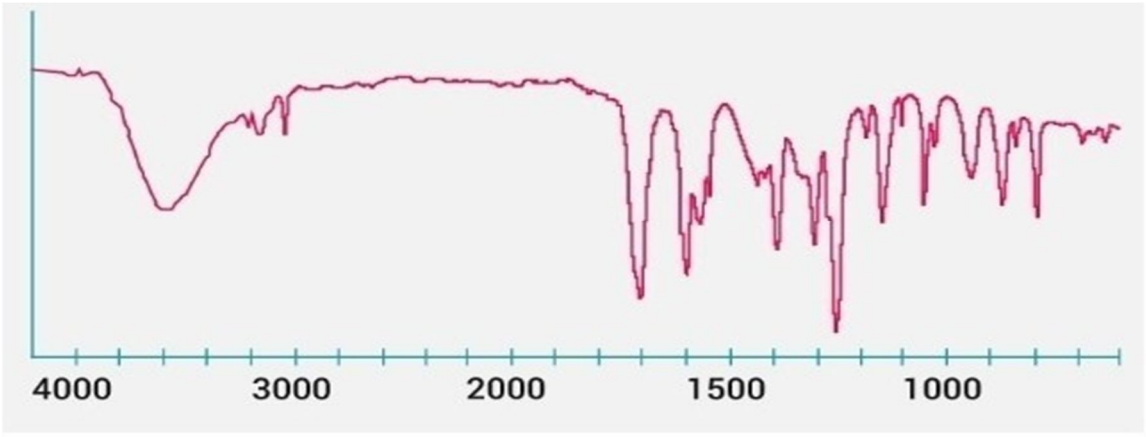


4) IR Spectroscopy Instrumentation

The instrumentation of infrared spectroscopy is illustrated below. First, a beam of IR light from the source is split into two and passed through the reference ant the sample respectively.

Now both of these beams are reflected to pass through a splitter and then through a detector. Finally, the required reading is printed out after the processor deciphers the data passed through the detector.

5) Graph of the IR Spectrum



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C. TLC Densitometric Method

Nasr Mohamed A.et al.[4] developed TLC Densitometric method for estimation of Indacaterol in bulk drug and capsules.

1) Apparatus

- Camag-Linomat 5 auto sampler (Switzerland) was used for data acquisition. The system is equipped with a deuterium and halogen tungsten lamp as a radiation, while a 100 µL syringe (Hamilton, Bonaduz, Switzerland) was used for sample application, the scanning mode is absorbance mode, the slit dimension is 3 mm × 0.45 mm, the scanning speed is 20 mm s–1.
- Pre-coated TLC plates, silica gel 60 GF254 (20 × 20 cm), (Fluka chemie, Switzerland).

2) General Procedure

Preparation of standard solutions

A standard solution of indacaterol (1mg/ml) was prepared by dissolving 100 mg of the drug powder in 50 ml of methanol and the volume was completed to 100 ml with the methanol. Working solution of (100 µg/ml) was prepared from the stock solution by suitable dilution with methanol.

Chromatographic condition for TLC-densitometric method

Analysis was performed on pre-coated 20 × 10 cm TLC aluminum silica gel 60 GF254 plates. Samples were applied to the plates using Hamilton micro syringe (50-µL). Plates were spotted 1 cm apart from each other and 1 cm apart from the bottom edge. The chromatographic tank was presaturated with the mobile phase for 20 min, then developed by ascending chromatography using (60% Methanol: 30%ethylacetate: 10%water by volume) as a mobile phase. The plates were air dried and the spots were scanned at 260 nm with CAMAG TLC scanner. The produced bands were scanned under deuterium lamp at 260 nm. Concentration of indacaterol in the produced bands was appears as peak areas in the TLC-densitometric chromatograms. The produced peak areas were plotted versus analyte concentrations and the regression equation of the linear relation was computed.

Linearity and construction of calibration graphs

Into a 10 mL volumetric flask, aliquot of standard solution equivalent to (1-6 mg) of indacaterol was transferred and diluted to volume with methanol. So the flasks contain (100-600) µg/mL, 10µL of solution was applied to a TLC plate following the above mentioned specific chromatographic conditions and scanned at 260 nm.

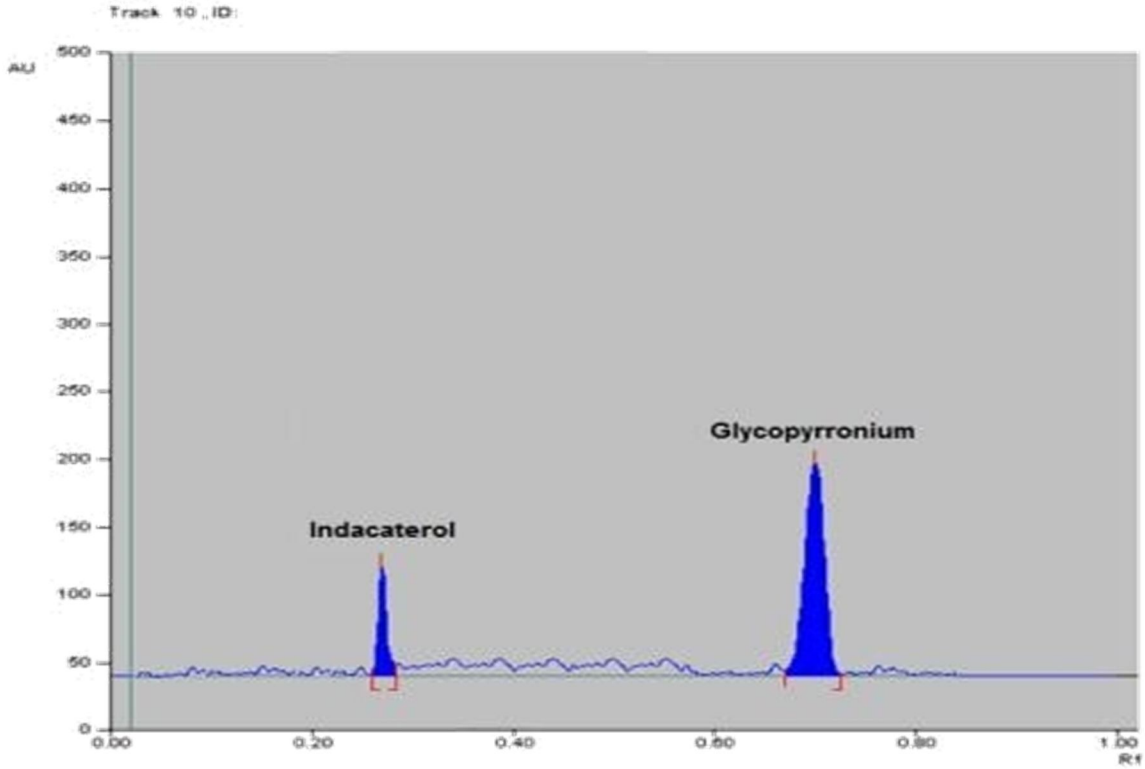


Fig: Two-dimensional TLC densitogram of indacaterol at 3 µg/band

3) Results and Discussion

Calibration data for determination of indacaterol by the proposed TLC-densitometric method:		
Calibration parameters		TLC-densitometric method
Wavelength (nm)		260
Linearity range (µg/band)		1-6
- Regression Equation -Slope (b) -Intercept (a)		*y= b x+ a* 701.528 274.066
Coefficient of determination (r^2)		0.9997
Accuracy (%R)*		99.67
Precision (%RSD)*	Repeatability	0.869
	Intermediate precision	0.963
LOD (µg/band)		0.108
LOQ(µg/band)		0.328

**Average of three replicates determinations of three concentrations (1, 2, and 4) µg/band of indacaterol.*

4) Conclusion

In this study, TLC-densitometric procedures has been developed and validated for the determination of in pure form and in their pharmaceutical preparation. The developed method is time saving where many bands can be run at the same time. This method is also economic since a small quantity of mobile phase as a developing system was used unlike HPLC procedures. Furthermore, this TLCdensitometric procedure can replace the HPLC method when HPLC requirements are unavailable.

Finally, we can conclude that the described TLC- procedure can be used in routine analysis of indacaterol in pure forms and pharmaceutical dosage form.

D. High Performance Liquid Chromatography method

Y. A. Salem et al.[5] Developed high performance Liquid chromatography for determination of Indacaterol adopting Ultraviolet and Fluorescence Detection in pharmaceutical formulation as well as in bulk.

1) 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) 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).

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3) Standard Stock And Working Solutions

Stock solution of 100 µg/mL of Indacaterol (IND) was prepared by dissolving 10.0 mg of Indacaterol (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.

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 (25ºC) 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.

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.

4) Results and Discussion

a) 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 1 and 2 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.

1.1 The 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.

1.2 The mobile phase

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). 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.

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 unretained 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.

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.

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 1.

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, rosuvastatin, 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.1 & 2.

b) 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.

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 2).

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 (Sy/x), intercepts (Sa), and slopes (Sb), and small values of the percentage relative standard deviations and the percentage relative errors (Tables 2). These data proved the linearity of the calibration graph.

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

LOQ and LOD were calculated according to ICH Q2 (R1) recommendations[6] using the following equations:

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

Where, Sa = 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.

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 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.

Statistical analysis 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 4 and 5) ,since the calculated values did not exceed the tabulated ones.

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 6).

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 5.

2.7 System suitability test (SST)

Evaluation of SST parameters was performed during the development and optimization of the method (Table 1). 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. SST parameters include tailing factor (*T*) and column efficiency (number of theoretical plates, *N*).

c) 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 .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 7. These control experiments eliminate the interference due to interactions of other constituents encountered in the system or caused by the bulk production.

5) Conclusion

The proposed method applies a validated HPLC to determine IND in pure form and in its capsule dosage 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.

Tables

Table 1. Optimization of the chromatographic conditions for the determination of IND by the proposed HPLC method using UV and fluorescence detection.

Parameter		Peak area		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)		6200	1050.5	1710.8	1.05	1.08
	(70:30)		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(tR/ Wh/2)^2 tR 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 Wh/2 is the peak width at the half height

Table 2. 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}
Sy/x	0.041	1.0×10^{-3}
Sa	0.028	5.0×10^{-4}
Sb	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 3. Accuracy and precision data for the determination of the IND by the proposed HPLC method.

		UV detection concentration (g/mL)			Fluorescence detection concentration (g/mL)		
		3.0	9.0	12.0	0.5	1.0	2.0
- day	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
- day	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 4. 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 (g/mL)		% fou		Conc. taken (g/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
			100.7	99.54			99.89	99.74
$\bar{X}$ ± 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 5. 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 * (g/mL)		% found		Conc. taken (g/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 6. Robustness testing for the assay of Indacaterol by the proposed HPLC method:

Content of Indacaterol using ultraviolet and fluorescent detectors										
Parameter	UV detection					Fluorescence detecion				
	Sample number					Sample number				
	1	2	3	Mean	%RSD	1	2	3	Mean	%RSD
Flow Rate (m L/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

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Table 7. RP-HPLC method for determination of IND using UV and Fluorescence detection applying the standard addition method:

Method	Capsule* concentration (g/ mL)	Concentration of added standard (g/ 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

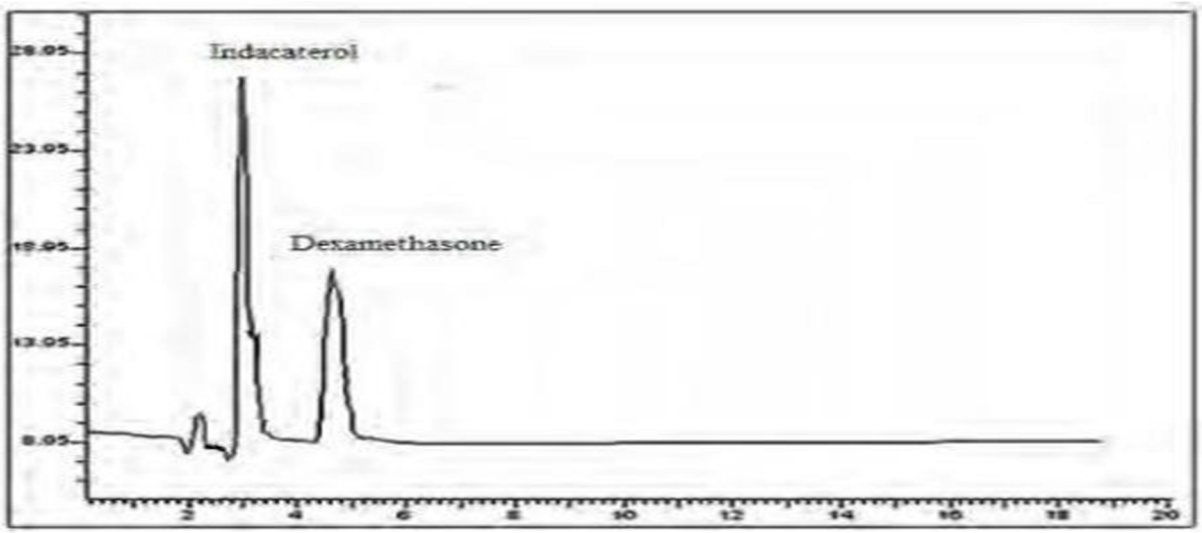


Fig. 1: Typical chromatogram for the separation of Indacaterol (12.0 µg/mL, 3.0 min) and dexamethasone (30.0 µg /mL, 4.6 min) using Rp-HPLC with ultraviolet detection at 259 nm and C18, pre-packed column RT (150 mm × 4.6 mm ID, Lichrosorb® RP-18 (5 µm particle size). The mobile phase composition was 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.

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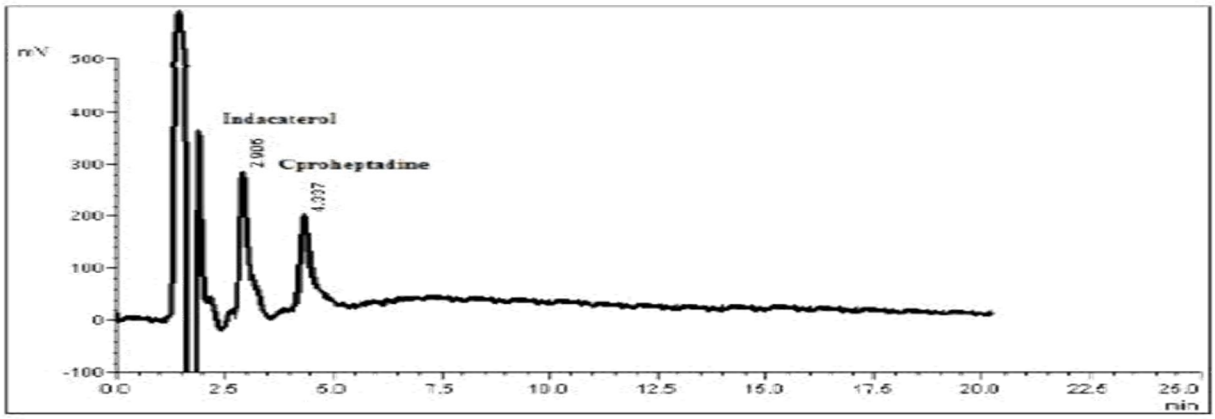


Fig. 2: Typical chromatogram for the separation of Indacaterol(5.0 µg/mL, 2.9 min) and cyproheptadine (5.0 µg /mL, 4.3 min) using Rp-HPLC with fluorescence detection (λex/em: 258/421 nm) and C18, pre-packed column RT. The mobile phase composition was acetonitrile: 0.05 M acid hydrogen phosphate buffer containing 0.3 % TEA (40: 60 %, V/V) adjusted to pH 3.0 using 0.02 M OPA.

E. Capillary Electrophoretic Method

Tufan G et al.[7] developed validated Capillary Electrophoretic method for the determination and validation of Indacaterol (IND) using an internal standard in capsules.

- **Apparatus:** All CE separations were conducted on a Capillary Electro- phoresis 1600 system with diode array UV detector (Agilent Technologies, G1600 A, Oregon, USA). Electrophoresis was performed in fused silica capillaries of 75 mm i.d. and of effective length of 50 cm and total 57 cm long (Agilent). Gas bubbles from all solutions and samples were removed by ultrasonic bath Sonorex (Bandelin, Berlin, G) and they were then centrifuged in a 4000 rpm speed centrifuge (Sigma, 1-6P Laboratory Centrifuge). The solutions pH was measured using a model pH/Ion meter- 720A with an Orion 71-03 glass electrode (ThermoOrion Beverly, MA 01915-6199, USA). All of the buffers and sample solutions were filtered through a regenerated cellulose (RC) membrane filter 0.45 mm prior to analysis (La- Pha-Pack, Rockwood, TN, USA).
- **Solution and sample preparation:** A stock solution (4.92×10^{-4}mol L^{-1}) was prepared by dissolving 25 mg of IND in 100 mL MeOH. Serial dilutions were performed with the 10% (v/v) MeOH/water mixture to obtain the appropriate concentration range. The standard solutions were stable for at least two weeks if kept in a refrigerator at +4°C. 15.2 mg methyl paraben (IS) was dissolved in 30 mL of MeOH and was then diluted with ultrapure water up to 100 mL. A stock sodium tetraborate buffer solution (100 mmol L^{-1}) was prepared, and then relevant running buffer solutions were obtained from this solution. The pH of the solution was adjusted to a desired value with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH. This was prepared daily by mixing appropriate volumes of stock buffer solutions, water and MeOH in order to adjust the pH to the desired value. Each inhalation powder hard capsule (Arcapta®) contains 194 µg of indacaterol, equivalent to 150 µg of indacaterol and certain inactive ingredients, such as lactose monohydrate and gelatine. Ten capsules were accurately weighed individually taking care to preserve the identity of each capsule. The contents of each capsule were removed. The emptied shells were then individually accurately weighed, and the net weight of contents for each capsule was calculated by subtracting the weight of shell from each respective gross weight. The drug substance content of each capsule was calculated from the net weight of the individual capsule content. The drug substance content of each capsule (25.3 mg of Arcapta®) was transferred into a small flask and extracted with 3 mL of MeOH. The mixture was centrifuged at 4000 rpm for 5 min and then filtered through a 0.45 mm membrane filter (RC). A 0.1 mL aliquid of the sample solution was diluted to 1 mL with water. Then 30 mL of IS at 1.05×10^{-3} mol L^{-1} was added. Finally, it was injected through the CE capillary.
- **CE conditions:** The system was thermostated at 25°C and the new capillary was conditioned by flushing at 9.35×10^{4} Nm^{-2} sequentially with 1.0 mol L^{-1} NaOH for 15 min, with 0.1 mol L^{-1}NaOH for15 min, water for 15 min and running buffer for 15 min. Between two consecutive analyses, the capillary was flushed sequentially with distilled water for 2 min, 0.1 mol L^{-1} NaOH for 1 min, and distilled water for 3 min, and finally with a running buffer for 5 min prior to use. All buffers and sample solutions were filtered through a 0.45 µm membrane filter prior to the analyses. Before injection through the capillary of CE, all solutions and samples were sonicated for 5 min. These were injected through the capillary for 10 s using a hydrodynamic injection mode applying low-pressure 5×10^{3} N m^{2} from the anodic side.

Signals were detected at 200 nm and the migration times were 4.84 and 6.47 min for IND and IS, respectively.

1) Method Validation

The method was validated according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures.

The results were evaluated using a rate of peak normalization (rPN) calculated by dividing the peak normalization value of IND into the peak normalization of IS [rPN = PNIND/ PNIS]. The peak normalization values for IND and IS were found by dividing their individual areas into their retention times IND [PNIND =Peak areaIND/Retention timeIND] and IS [PN IS = Peak areaIS/Retention timeIS]. During the experimental evaluations, the related parameters were investigated against PN or rPN.

- **System suitability of the method**

The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. The parameters were tested for basic analytical parameters including capacity factor, resolution, tailing of the peak, theoretical plate, retention time and percentage of repeatability and were evaluated using Agilent Software for system suitability of developed method.

- **Precision**

The validation of tests for assay and for quantitative determination of impurities includes an investigation of precision. The precision of the method was calculated by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying the samples of the same concentrations for five times over three successive days (n =6). Standard solution of IND (0.98 x 10^{-5} mol L^{-1}) and constant IS solution (3.06 x 10^{-5} mol L^{-1}) were used for the precision experiments. Intermediate precision was found by comparing the experiments on three different days.

- **Calibration tests**

Calibration studies were carried out by preparing three sets and five dilutions (n =5) for each set in the range of 4.96 x 10^{-6} mol L^{-1} and 3.94 x 10^{-5} mol L^{-1}. These were then injected through the capillary consecutively for three days (intra-days). Calibration curves were found by linear regression analysis using the least square method. Slope, intercept, correlation coefficient, standard deviation of regression equation for the calibration equations, and confidence limits (for $P < 0.05$) were calculated both intra-day and inter-day. Certain analytical parameters, such as LOD and LOQ values, were found by computing the integrated peak from the CE electropherogram. LOD and LOQ values were calculated [(standard deviation of regression equation)/(slope of regression equation)] by multiplying by 3.3 and 10, respectively.

- **Accuracy of the method**

The accuracy of the method was tested by the standard addition method as stated: Definite amounts of standard IND (0.598 mL; 1.201 mL and 1.799 mL of 4.92 x 10^{-4} mol L^{-1}) were spiked into approximately 25.3 mg of Arcapta®. It was extracted with 3 mL of MeOH. The mixture was centrifuged at 4000 rpm for 5 min and then filtered through a 0.45 µm membrane filter (RC). An aliquid of 0.1 mL of the sample solution was diluted to 1 mL with water (Final concentrations were
8.18 x 10^{-6}, 1.41 x 10^{-5}, and 1.84 x 10^{-5} M, respectively) and then 30 µL of IS at 1.05 x 10^{-3} mol L^{-1} was added. It was then injected through the CE capillary.

Determination of IND in pharmaceutical formulation

IND was determined in the pharmaceutical formulation of Arcapta® inhalation powder hard capsule dosage form by the developed CE method. The sample was weighted and prepared as in the section *'Solution and sample preparation'*.

2) Results and Discussion:

- **Optimization of the method**

Indacaterol (IND) has two pKa values of 8.5 and 9.7. Even if EOF is still strong at pH 7.5 and higher pH values, IND moves with EO peak at 7.5. At higher pH of 7.5, IND moves after EOF. The best peak shape was observed at pH 10.0 regarding efficiency and peak width. Retention time of IND was around the same at pH 8, 9, 9.5, 10.0 and 10.5. At pH 10.5, the IND peak was broadened. At pH 10.0, the value of rPN is the highest (Fig. 2). Thus, pH 10 buffer was selected as an optimum pH value. IND is completely in anionic form around pH 10.

Sodium phosphate and sodium tetraborate buffers were tried as alkaline buffer. Sodium phosphate was not used due to high current, and a high noise signal in the electropherograms. In further optimization studies, sodium tetraborate buffer was used as the running buffer in this study.

In the CE analysis, the addition of organic modifier to the running buffer changes both the zeta potential and electrolyte viscosity. The organic modifier can change selectivity by changing electroosmotic flow mobility. For this purpose, methanol and acetonitrile as organic modifiers for the running buffer, consisting of sodium tetraborate, were tried. Since the IND solution was not dissolved entirely in acetonitrile, the standard solutions of IND and the sample were prepared by dissolving them in methanol and this was used as an organic modifier throughout the study.

The concentrations of sodium tetraborate of (10-25) mmol L^{-1}, percentages of organic modifier MeOH of 10-20, % and pH ranges of 9.5-10.5, applied potentials of 10-25 kV, injection times on 5-10 s at 5 x 10^3 N m^{-2} and the effect of wavelength (between 200 nm and 260 nm) were examined to determine the optimum conditions. Optimal conditions were determined in the viewpoint of peak shape, resolution, sensitivity and retention time. These were a running buffer consisting of 20 m mol L^{-1} sodium tetraborate solution, 15% (v/ v) MeOH at pH = 10.0, 20 kV of applied potential, 10 s at 5 x10^3 Nm^{-2} injection time, 200 nm wavelength and a 25°C fixed temperature. Certain internal standards were tried to determine a suitable IS to increase the repeatability and sensitivity of the developed method. Nicotine amid, phenobarbital, methyl paraben, ethyl paraben, propyl paraben and butyl paraben were tried as the candidate for internal standard. Methyl paraben was a suitable IS for this system and appeared in a reasonable time.

The electropherogram of standard IND (0.98 x 10^{-5} mol L^{-1}) and IS (3.06 x 10^{-5} mol L1) solution in the optimum conditions is demonstrated in Fig. 2.

Under optimum conditions, IND and IS migrated in 4.84 (RSD 0.39%) and 6.47 (0.42%) minutes, respectively. The mean electrophoretic mobility toward the anode (m^2/sV) of IND and IS was calculated as -1.63 x 10^{-4} (RSD: 0.98%) and -2.89 x 10^{-4} (RSD: 1.99%), respectively (n = 3).

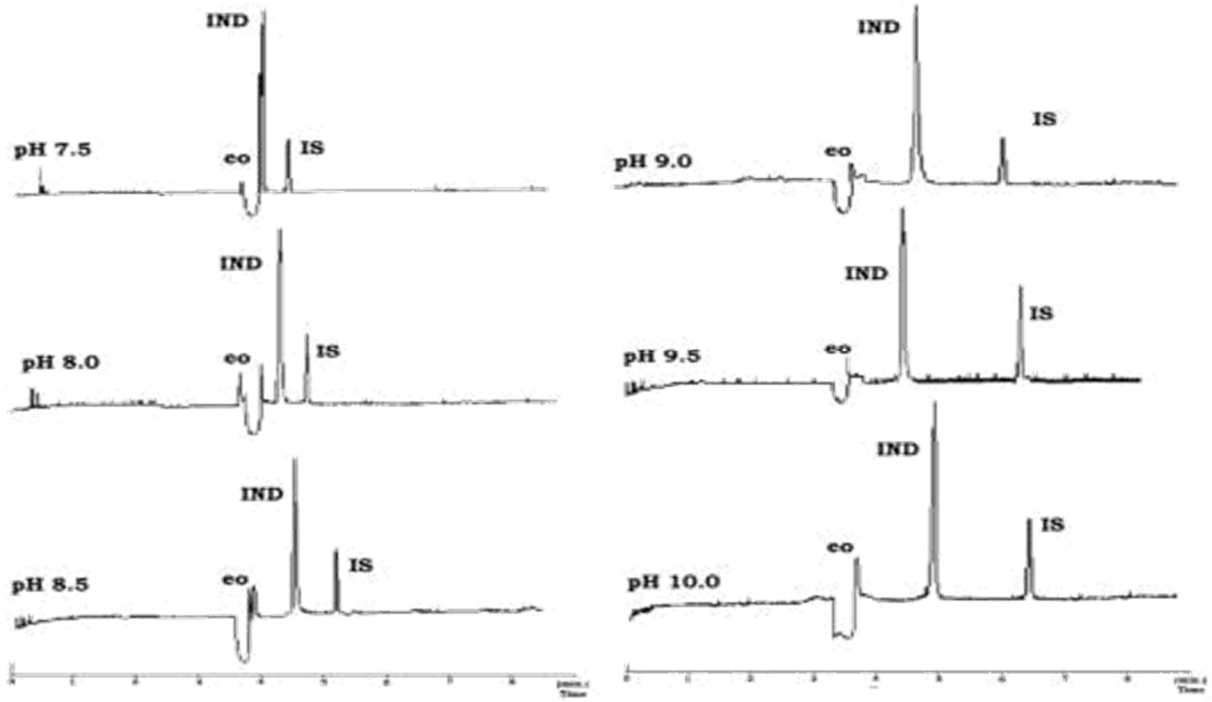


Fig. 2 - The electropherograms of IND (4.92 £ 10¡5 mol L¡1) and IS (3.06 £ 10¡5 mol L¡1) in the employment of 20 mmol L¡1 sodium tetraborate buffer, 15% (v/v) MeOH, at different pH values in the use of 20 kV of applied potential, 10 s at 5 £ 103 N m¡2 of injection time, 200 nm of wavelength and 25°Cof fixed temperature.

- **Method validation**

The solution containing 0.98 x 10^{-5} mol L^{-1} IND and IS (3.06 x 10^{-5} mol L^{-1}) was consecutively injected through the capillary to determine efficiency. Under optimum conditions, the obtained system suitability parameters are given in Table 1. The system suitability values confirm that the determination of IND can be achieved successfully. The experimental values obtained from the results of examination of precision for IND are presented in Table 2. As can be seen from Table 2, RSD values of 0.91-1.18% show good intra-day precision. Inter-day results were calculated from the three days of inter-day experiments obtaining on an RSD value of 1.04%. The RSD% values show that the method is precise and acceptable from an analytical point of view. The calibration curve is given for

IND in the range of 0.49 x10^{-5} mol L^{-1} (2.50 µg mL^{-1}) and 3.94 x 10^{-5} mol L^{-1} (20.0 µg mL^{-1}) with R = 0.9993 for inter-day and, LOD and LOQ values in Table 3. As can be seen from Table 3, the calibration equation is linear (rPN =7.55 x 10^5 CIND -0.11) on the basis of inter-day results, with a good correlation coefficient (0.9993). The LOD and LOQ values were calculated to be 2.18 x 10^{-8} mol L^{-1} (0.011 µg mL^{-1}) and 7.25 x10^{-8} mol L^{-1} (0.037 µg mL^{-1}) for IND, respectively, on an inter-day basis. The results are reasonably low for L

Table 1 – System suitability parameters of IND in the optimum conditions.

Parameters	Observed value (IND)	Observed value (IS)
Migration time (t as min)	4.84	6.47
Capacity factor (k′)	0.33	0.77
Tailing factor (T)	1.23	1.41
Theoretical plates (N)	2.01×10^5	2.51×10^5
Resolution (R_s)	34.35	
Separation factor (α)	1.34	

OD and LOQ for the determination of IND.

Table 2 – The results of intraday and inter-day precision of IND (employing 0.98×10^{-5} mol L^{-1} IND and 3.06×10^{-5} mol L^{-1} IS) computed by the rPN vs concentration of IND ($rPN = PN_{IND}/PN_{IS}$).

	I. Day (n = 6)[a]	II. Day (n = 6)[a]	III. Day (n = 6)[a]	Inter-day (n = 18)[a]
$\bar{X}$[b]	0.60	0.59	0.59	0.59
s[c]	0.01	0.01	0.01	0.01
RSD%[d]	1.18	1.06	0.91	1.04
CL[e]	0.01	0.01	0.01	0.01

[a] n is the number of experiments.
[b] $\bar{X}$ is the mean ratio of peak-normalization.
[c] s is the standard deviation of the mean response.
[d] RSD% is the relative standard deviation as percent.
[e] CL is confidence limits, $\left(\frac{ts}{\sqrt{n}}\right)$.

Table 3 – Calibration elements for IND in the range of 0.49×10^{-5} mol L^{-1} (2.5 µg mL^{-1}) and 3.94×10^{-5} mol L^{-1} (20.0 µg mL^{-1}) in optimum condition.

	I. Day, n = 6	II. Day, n = 6	III. Day, n = 6	Inter-day, n = 18
m[a]	7.62×10^5	7.45×10^5	7.59×10^5	7.55×10^5
n[b]	−0.12	−0.11	−0.11	−0.11
R[c]	0.9991	0.9994	0.9991	0.9993
s_m[d]	0.04	0.03	0.04	0.03
CL[e]	±1456	±1202	±1442	±1290
LOD[f] (mol L^{-1})	2.16×10^{-8}	2.21×10^{-8}	2.16×10^{-8}	2.18×10^{-8}
LOQ[g] (mol L^{-1})	7.19×10^{-8}	7.36×10^{-8}	7.21×10^{-8}	7.25×10^{-8}

[a] m is slope.
[b] n is intercept.
[c] R is correlation coefficient.
[d] s_m is the standard deviation of calibration curve, $\left(s_m = \sqrt{\frac{s_r^2}{s_{xx}}}\right)$, s_r is standard deviation of regression.
[e] CL is confidence limits, $\left(\frac{ts_m}{\sqrt{n}}\right)$.
[f] LOD is limit of detection.
[g] LOQ is limit of quantification.

The accuracy of the method was examined by the standard addition method as stated in the experimental section of 'Accuracy of the Method'. The results are presented in Table 4.

Table 4 – The results of accuracy of IND (n = 6) by the standard addition method as stated in 'Accuracy of the Method' of the experimental section.

Added IND, (mol L^{-1})	Found IND, (mol L^{-1})	Recovery% (RSD%)
0.98×10^{-5}	0.97×10^{-5}	99.09 (0.21)
1.97×10^{-5}	1.94×10^{-5}	98.84 (0.01)
2.95×10^{-5}	2.91×10^{-5}	98.79 (0.80)

The calculated recoveries % (98.79 - 99.09%) demonstrate that the proposed method has excellent accuracy (Table 4). The recovery ranges % were in agreement with accepted criteria, which were in the range of 85 - 115%.

The ability of the method to remain unaffected by small but deliberate variations in the optimization parameters was measured via robustness to test the method's reliability during usage. In the robustness experimental design, a single variable has been changed at a time. For this purpose, certain parameters such as pH, sodium tetraborate concentration, MeOH percentage, applied voltage, wavelength, column temperature and injection time has been changed and the results have been compared than those in optimum conditions. The parameters concerning robustness are given in Supplementary Table 1. The values of standard error of the mean (SE < 1) for robustness parameters demonstrate that the developed method is highly reliable. All of the small changes are in an acceptable range regarding RSD% value < 2 for each parameter and it can be seen that the method is highly robust.

3) Application of the method to IND in the pharmaceutical preparation

The analysis of the IND in Arcapta® capsule dosage form was realized as stated in the experimental section of 'Preparation of Sample' and 'Determination of IND in pharmaceutical formulation'. A typical electropherogram of the Arcapta® capsule dosage form has been shown in Fig. 3.

The output of the experiments was evaluated and rPN values were calculated. The active substance,

IND, was computed from the calibration equation. The results and evaluations are shown in Table 5. As can be seen from Table 5, the determined IND amount was 193.45 mg of indacaterol with a relative error of 0.28% (certified IND value: 194 mg of indacaterol equivalent to 150 mg of indacaterol) in Arcapta® capsule dosage form. Since IND is a new drug, any monograph in the pharmacopeias has not yet been reported. The acceptance criteria allows for a 15% deviation. The deviation of our result for IND (0.28%) is within limits. The relative standard deviation (RSD%) of the drug substance in the final dosage units should not be more than 2% according to the Pharmacopeia. The RSD% value (0.37) in our study is smaller than this value (2%) (Table 5). As a result, the RSD% and the absolute error values are within limits. These considerations show us that the presented method is valid and reliable.

Supplementary Table 2 summarizes the analytical performance of our CE method compared to other methods for the determination of IND. Among these methods, HPLC-MS provides the lowest LOD as well as fluorescence for IND. Unfortunately, the method is expensive, not portable, requires an experienced technician, and has a matrix effect. The CE method is comparable with the HPLCFluorescence method. HPLC-UV and UV-Vis methods possess higher LOD values than those for CE method.

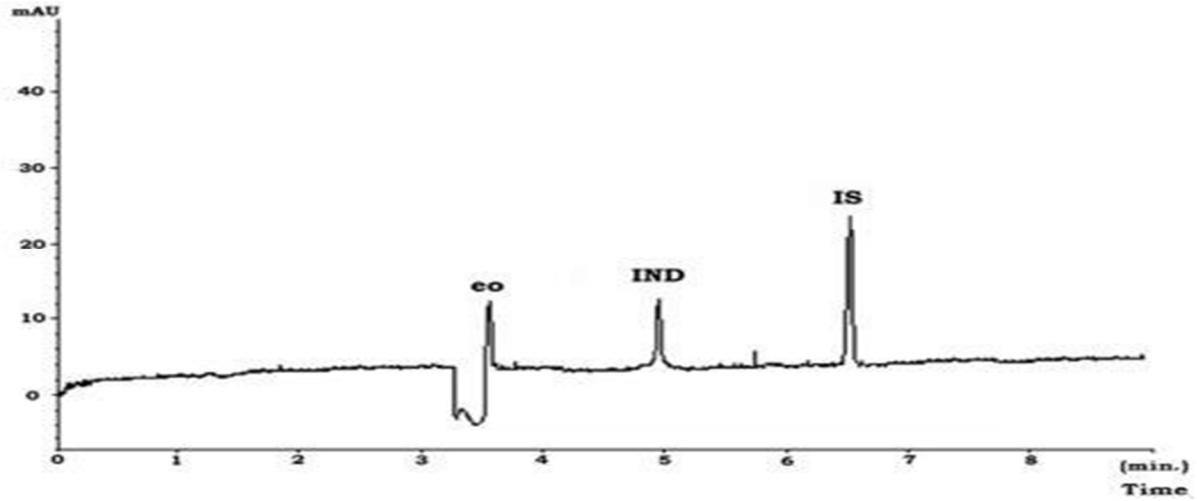


Fig. 3 - A typical electropherogram of Arcapta® capsule on optimum conditions

Table 5 – The results of IND in Arcapta® inhalation powder hard capsule by the CE method (Certified value: 194 μg of indacaterol maleate equivalent to 150 μg of indacaterol on each capsule Arcapta®), (n = 6).[a]

Parameters	Found (Relative Error, %)[b] (μg capsule^{-1})
$\bar{X}$[c]	193.45 (0.28%)
S[d]	0.72
RSD%[e]	0.37
CL[f]	0.72

[a] n is the number of experiments.
[b] Relative error is the magnitude of the difference between the exact value and the found value divided by the magnitude of the exact value as percent.
[c] $\bar{X}$ is the mean by regarding ratio of peak-normalization.
[d] s is the standard deviation of the mean response.
[e] RSD% is the relative standard deviation as percent.
[f] CL is confidence limits, $\left(\frac{s}{\sqrt{n}}\right)$.

IV. CONCLUSION

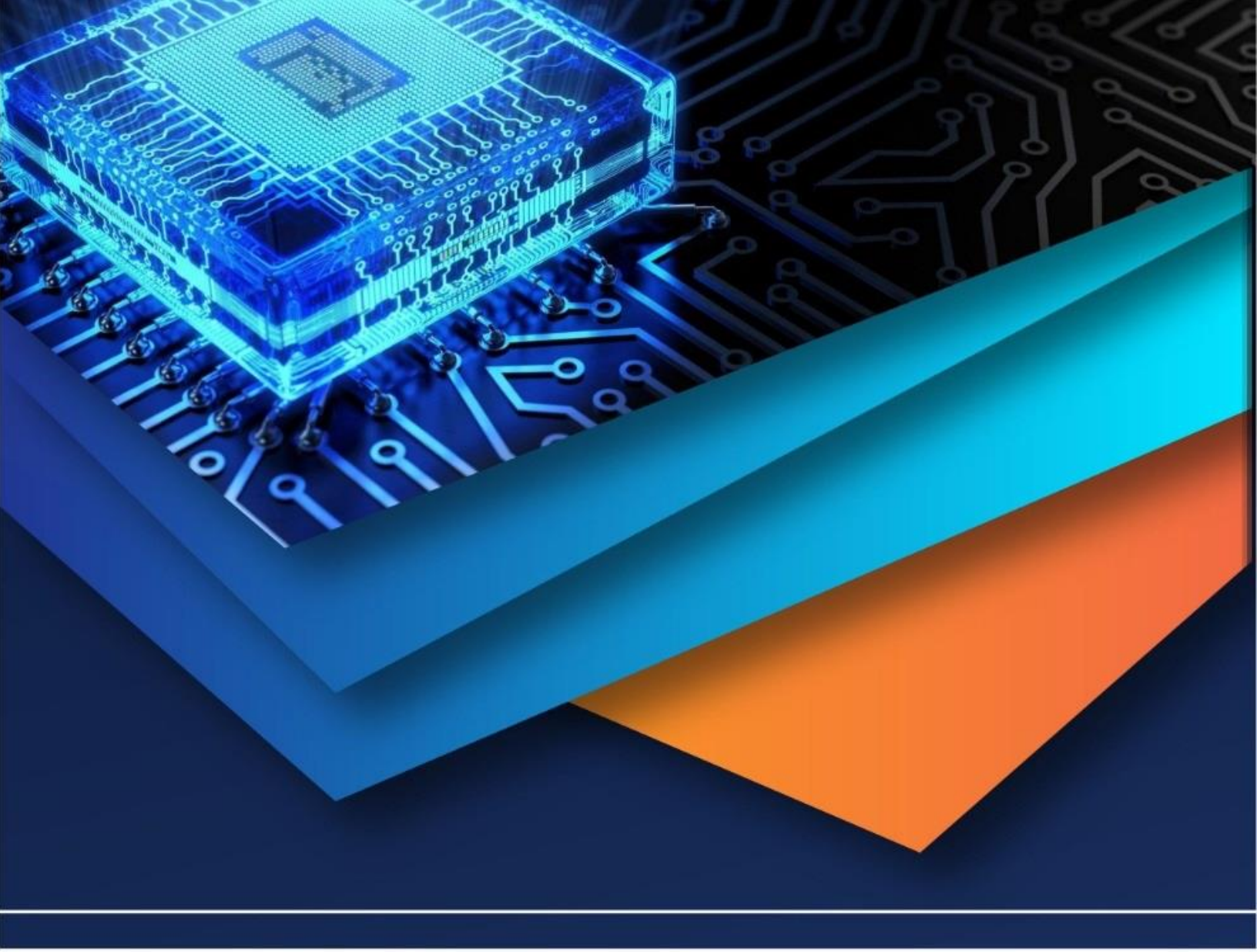
A rapid, accurate, selective, reliable and environmentally friendly new capillary electrophoretic method for the determination of IND was developed and is validated in this study. The method is simple, easy to apply and IND was determined within 8 min. The method is also cheap and has many advantages over conventional chromatographic techniques, including reduction in the use of organic solvents, small sample volume, and increased efficiency and resolution. To the best of our knowledge, this is the first CE study concerning the determination of IND.

The method has been validated with respect to precision, linearity range and accuracy, LOD, LOQ, specificity and robustness. All the system suitability parameters gave good results. The proposed method has been successfully applied to the analysis of IND in the pharmaceutical preparation of Arcapta® capsule dosage form.

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