DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF DRUG SUBSTANCES, IMPURITIES AND RESIDUAL SOLVENTS BY RP-HPLC/GC

Thesis submitted in fulfillment of the requirements for the Degree of

Doctor of Philosophy

In

Pharmaceutical Technology

Submitted by

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the PhD thesis entitled **"Development and validation of chromatographic methods for the determination of drug substances, impurities and residual solvents by RP-HPLC/GC"**, submitted by **Mr. Md. Faisal Hossain** at **University of Dhaka, Bangladesh**, and is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Development and validation of chromatographic methods for the determination of drug substances, impurities and residual solvents by RP-HPLC/GC"**, submitted at **University of Dhaka, Bangladesh**, is an authentic record of my work carried out under the supervision of **Professor Dr. Abu Shara Shamsur Rouf, Professor Dr. M. O. Faruk Khan and Professor A B M Faroque**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Theses.

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DECLARATION OF THE COPYRIGHT AND OWNERSHIP

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ABSTRACTS OF THE THESIS

Department of Pharmaceutical Technology
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THESIS TITLE

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Submitted by

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ABSTRACTS OF THE THESIS

1.0 OBJECTIVE

The objective of this research work are as follows

1. To develop and validate stability indicating test method as per ICH guidelines for determining the content of drug substance by RP-HPLC to establish the quality of the products with a consistent results.
2. To develop and validate stability indicating test method as per ICH guidelines for the determination of impurities, and degradants by RP-HPLC to establish the quality of the product with a consistent results.
3. To develop and validate RP-HPLC test methods for the determination of physicochemical properties (potency, solubility, stability, pKa) of a novel drug lead.

2.0 SCOPE

The developed methods can be used in research and development and in quality control laboratories in pharmaceuticals industries to characterize drug substances and drug products during the process of drug discovery and development by implementing the modern quality by design (QbD) approaches.

3.0 LABORATORY INVOLVE

1. College of Pharmacy, Southwestern Oklahoma State University, USA.
2. Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka.

4.0 SUMMARY

The purpose of this study was to develop and validate chromatographic methods to characterize pharmaceutical drug substance/product in terms of potency/purity, impurities, solubility, stability, and ionization constant. Reverse phase-high performance liquid chromatography (RP-HPLC) technique was mainly employed to develop and validate all chromatographic methods. Chapter I, of the thesis contains three parts which are introduction, analytical method development and analytical method validation based on RP-HPLC. Chapter II, therefore, describes the characterization of a novel anti-malarial drug lead Cyclen Bisquinoline using RP-HPLC methods. Chapters III and IV describes a development and validation of RP-HPLC stability indicating assay and impurity test method for non pharmacopoeial products, Aceclofenac Tablet and Salmeterol Inhaler dosage forms, respectively. All the RP-HPLC methods developed and validated to determine the content and related substances in the pharmaceutical tablets dosage form is specific, precise, linear, accurate, and rugged enough, which can be used in research and development and in quality control laboratories in pharmaceutical industries. Methods developed for novel anti- malarial drug lead Cyclen Bisquinoline would be useful in implementing the modern Quality by Design (QbD) approaches for further development of the drug leads to characterize their purity, stability, metabolic stability, solubility and ionization constant.

5.0 ABSTRACTS

5.1 CHAPTER-II

Title:

Development and Validation of RP-HPLC methods to determine purity, solubility, stability and pKa of a novel antimalarial drug lead, Cyclen Bisquinoline.

5.1.1 Solubility Study

Abstract

The purpose of this study was to develop and validate a RP-HPLC method to evaluate the solubility of a novel antimalarial drug lead, CNBQ (free base; FB) and its HCl salt. Chromatographic separation of drug lead CNBQ, was successfully achieved on a C-18 column (4.6mm x 250mm, 5.0µm) in an isocratic separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with Phosphoric Acid in the ratio of (70:30, v/v). The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325 nm. Equilibrium solubility and stability of both FB and its salt were carried out in different mediums and samples were analyzed using RP-HPLC. The equilibrium solubility order of the FB is: DMSO > ethanol > pH 1 > isopropyl alcohol > tween 80 > propylene glycol > PEG 400 > water > pH 4.5 > pH 7.4 > pH 9.0 > pH 12.0; and that of salt is: water > propylene glycol > DMSO > ethanol > PEG 400. It is highly hydrophobic, however, salt formation improved its water solubility by approximately 370-fold.

5.1.2 Purity determination and stability study

Abstract

The purpose of this study was to evaluate the purity and stability of a novel antimalarial drug lead, CNBQ (free base; FB) and its HCl salt using reverse phase-high performance liquid chromatography (RP-HPLC). Chromatographic separation of drug lead CNBQ, was successfully achieved on a C-18 column (4.6mm x 250mm, 5.0µm) in a gradient separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with Phosphoric Acid. The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325nm.

Stability of both FB and its salt were carried out in different mediums and samples were analyzed using reverse phase-high performance liquid chromatography (RP-HPLC). The solutions of both form were stable in acid (0.1M HCl, pH 1.0 buffer), base (0.1M NaOH, pH 12.0 buffer), and water, but unstable in 0.3%H2O2. The hydrochloride salt form was found to be more pure and stable than the FB.

5.1.3 Metabolic (in vitro) stability study

Abstract

The metabolic stability of CNBQ was determined using human liver microsome (HLM) and specific cytochrome P450 enzyme (CYP2C8) taking the clinically used antimalarial drug chloroquine as a positive control. Chromatographic separation of drug lead CNBQ, CQ and their metabolites were successfully achieved on a C-18 column (4.6mm x 250mm, 5.0μm) in an isocratic separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with phosphoric acid in the ratio of (60:40, v/v). The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325nm. Peak purity data was obtained using photodiode array (PDA) detector. All the assays were conducted in 0.5M phosphate buffer at pH 7.4. In general the metabolic reaction was initiated by adding 1mM NADPH and 0.5 mg of enzyme. Incubations were done with time frequency of 0hr, 1hr, and 2hrs at 37oC and the reactions were terminated by adding acetonitrile in the equal amounts of the assay mixture taken. The samples were centrifuged for 15min at 10,000 x g at 4oC and an aliquot of the supernatant was subjected to analysis using HPLC as well as LC-MS to confirm the masses of the drug and/or metabolite (s), if any. While chloroquine was found to be metabolized in a predictable manner by both HLM and CYP2C8, the drug lead was metabolically stable at similar experimental conditions. This study demonstrated that the new drug lead is worth conducting further preclinical evaluations.

5.1.4 Determination of pKa values

Abstract

The purpose of this experiment was to evaluate and compare the pKa values of the poorly water soluble, polyprotic weakly basic drug lead, CNBQ. Three separate methods, pH-metric, UV-metric, and reverse phase-high performance liquid chromatography (RP-HPLC), were employed to determine the pKa values between 2.0-12.0 pH range. The acetate and phosphate buffers, in addition to methanol and acetonitrile as co-solvents and potassium chloride to maintain the ionic strength, were used as appropriate. In UV-metric method, the drug substance is dissolved in aqueous media eliminating any interference of a co-solvent for

measuring the pKa. Consequently, the pKa values obtained by the UV-metric method are considered accurate, as opposed to Potentiometric and RP-HPLC methods that require the use of co-solvents. Thus, through the utilization of UV-metric method three pKa values, 5.9, 6.6, and 8.7, were obtained for CNBQ. These studies would be useful to determine the pKa values of the related drug leads under development.

5.2 CHAPTER-III

Title:

Development and validation of stability indicating assay test method to determine the content of aceclofenac in the pharmaceutical tablet dosage forms by RP-HPLC method.

Abstract

The purpose of this study was to develop and validate a versatile, rapid and precise stability indicating reversed phase HPLC test method to determine the content of aceclofenac (ACF) in the pharmaceutical tablet dosage form by RP-HPLC. ACF is decomposed under acidic and basic conditions, oxidative stress, in presence of light and also in different thermal stresses conditions. Chromatographic separation of ACF and its major and minor degradation products were successfully achieved on a C18 (250 mm length × 4.6 mm i.d., 5 μm particle size) column in an isocratic separation mode with mobile phase consisting of 0.07% of orthophosphoric acid and acetonitrile in the ratio of (68 : 32, v/v) at pH 7.0 ± 0.05, and the flow rate was maintained at 1.2 mLmin-1 and the effluent was monitored at 275nm. Peak purity data of ACF was obtained using photodiode array detector (PDA) in the stressed sample chromatograms, which demonstrated the specificity of the method for the estimation of ACF in presence of degradation products. The method was validated with respect to linearity, precision, accuracy, selectivity, specificity, and ruggedness to assay ACF in tablets. The method was linear over the concentration ranges of 160–240 μgmL-1 (R2 = 0.9993). Degradation products resulting from stress studies did not interfere with the detection of ACF and the assay method is thus stability indicating.

5.3 CHAPTER-IV

Title:

Development and validation of the stability indicating test method to determine the content of salmeterol xinafoate and its organic impurities in pharmaceutical pressurized inhalation dosage form by RP-HPLC.

Abstract

The purpose of this study was to develop and validate a stability indicating RP-HPLC test method to determine the content of drug substance and its organic impurities of salmeterol xinafoate (SX) in inhaler dosage form. Chromatographic separation of SX and its known impurities were successfully achieved on a stainless still column packed with C18 column (250 mm × 4.6 mm, 5 μm) in a gradient separation mode with mobile phase consisting of phosphate buffer (0.015 M KH2PO4 at pH 6.8 ± 0.05) and methanol. Flow rate was maintained at 1.0 mL.min-1 and the effluent was monitored at 278nm. As per ICH guidelines, the method was validated with respect to suitability, specificity, linearity, precision, accuracy, and robustness. Specificity of the method in presence of impurities and degradation products was evaluated by peak purity testing using photodiode array (PDA) detector. In order to check the peak purity, samples were kept under different stressed conditions (acidic, basic, oxidative, thermal and light) and were spiked with known impurities. The method was found to be linear (R2<0.99) over the concentration ranges of LOQ to 120% of specification level. The RP-HPLC method was also found to be accurate and precise with percentage recovery values between 98.0% to 102.0% and 90.0% to 110.0%, and percentage RSD values less than 2.0% and 10.0% for SX and its all impurities, respectively. Test sample solution is found to be stable up to 24 h at room temperature. Hence, this method can be used to quantify the amount of SX and its impurities in the drug product during product development and routine analysis.

TABLE OF CONTENTS

LIST OF TABLES

Chapter-III/Stability Indicating Assay Test method

Chapter-IV/Stability Indicating Assay and Impurity Test method

LIST OF FIGURES

1.0 OBJECTIVE

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- To develop and validate stability indicating test method as per ICH guidelines for determining the content of drug substance by RP-HPLC to establish the quality of the products with a consistent results.

- To develop and validate stability indicating test method as per ICH guidelines for the determination of impurities, and degradants by RP-HPLC to establish the quality of the product with a consistent results.

- To develop and validate RP-HPLC test methods for the determination of physicochemical properties (potency, solubility, stability, pKa) of a novel drug lead.

2.0 SCOPE

The developed methods can be used in research and development and in quality control laboratories in pharmaceuticals industries to characterize drug substances and drug products during the process of drug discovery and development by implementing the modern quality by design (QbD) approaches.

3.0 SUMMARY

The purpose of this study was to develop and validate chromatographic methods to characterize pharmaceutical drug substance/product in terms of potency/purity, impurities, solubility, stability, and ionization constant. Reverse phase-high performance liquid chromatography (RP-HPLC) technique was mainly employed to develop and validate all chromatographic methods. Chapter I, of the thesis contains three parts which are introduction, analytical method development and analytical method validation based on RP-HPLC. Chapter II, therefore, describes the characterization of a novel anti-malarial drug lead Cyclen Bisquinoline using RP-HPLC methods. Chapters III and IV describes a development and validation of RP-HPLC stability indicating assay and impurity test method for non pharmacopoeial products, Aceclofenac Tablet and Salmeterol Inhaler dosage forms, respectively. All the RP-HPLC methods developed and validated to determine the content and related substances in the pharmaceutical tablets dosage form is specific, precise, linear, accurate, and rugged enough, which can be used in research and development and in quality control laboratories in pharmaceutical industries. Methods developed for novel anti- malarial drug lead Cyclen Bisquinoline would be useful in implementing the modern Quality by Design (QbD) approaches for further development of the drug leads to characterize their purity, stability, metabolic stability, solubility and ionization constant.

4.0 CHAPTER I

4.1 INTRODUCTION

The process of drug discovery holds a significant place in the field of medicine and pharmacology. Basically, the compounds with drug-like properties have been a target to pharmaceutical companies for drug discovery and development. Early evaluation of compounds with drug-like properties will be helpful for speculation and recognition of potential challenges during the process of drug discovery and development. Characterization of drug substances for their physicochemical properties such as solubility, state, ionization constants, lipophilicity, potency, impurities, environmental stability, and metabolic stability, not only saves time but also makes the process economical. These evaluations have significantly decreased the attrition rate in drug discovery and development due to factors like suboptimal physiochemical parameters, pharmacokinetics, and bioavailability. Compounds with optimal physicochemical parameter that will ensure suitable biopharmaceutical properties should be selected for further discovery and development of the drug leads to clinically useful drugs.[1]

The solubility of drugs is an important parameter to be evaluated in the process of drug discovery and development, which can create obstacles during the development process. The solubility of a drug is referred as a maximum dissolved concentration in a provided condition. Besides the properties of a drug molecules and solvent, factors like polarity, ionization potential, size, form, and pH of the drug has a huge impact on the solubility.[2] Thermodynamically stable crystalline form will be more stable and hence less soluble whereas amorphous form will be less stable, more soluble, and prone to crystallization and degradation.[3] High Performance Liquid Chromatography (RP-HPLC) technique is an efficient way to determine the solubility of a drug substance.

Occurrence of solid-state phase transformation is very prominent during drug formulation development due to drug-excipient(s) interaction.[4-6] This phase transformation may have a huge effect on factors like dissolution, bioavailability, and

stability of the drug. Determination and quantification of phase transition and conformational changes hold significant value in drug discovery and development. The energy of phase transition, conformational changes, and melting point can be determined using differential scanning calorimeter.

Lipophilicity is a property of a molecule to get involve with the lipid phase and penetrate lipid bilayer to reach the therapeutic target site.[7] Lipophilicity has an immense impact on the factors like solubility, permeability and ADME properties.[2] The lipophilicity of the drug helps to determine its susceptibility for metabolism. More lipophilic drugs are more susceptible to metabolism.[8] The logarithms of partition coefficient (log *P*) and distribution coefficient (log *D*) of new chemical entities are commonly used to describe lipophilicity using 1-octanol as lipophilic phase and water as an aqueous phase. RP-HPLC technique is an efficient way to obtain log *P* values of new drug leads.[9] Determination of lipophilicity is a fundamental requisite during the early stages of the drug development processes.

Ionization constant (pKa), also known as acid dissociation constant, has been regarded as an important parameter in the process of drug discovery and development. Solubility of ionizable compounds is dependent on the pH of the solution. Thus, solubility, membrane permeability and absorption of drugs are correlated to ionization constant.[10] Metabolism of the drug has been massively affected by the fluctuation in pKa value due to modification in overall polarity and electron density of molecules.[11] The potentiometric titration, spectrophotometric titration, RP-HPLC, solubility profile, and capillary electrophoresis are well-recognized methods for the determination of the ionization constant.[12, 13]

One of the indispensable physicochemical properties of the compound that is to be considered during drug discovery process is screening of the stability of drug candidates. This screening provides overall stability of compounds in various pharmaceutical conditions and also helps to identify impending hurdles during development process.[2] International Conference on Harmonisation (ICH) guidelines require the parent drugs to be tested under stress conditions such as effect of pH, temperature, humidity, light, and oxidizing agents. Stability of compounds in solution

at different pHs is required to formulate the solution dosage forms. Oxidation being the most common degradation pathway for organic compounds. Thus, analysis of oxidative stability is one of the crucial steps in drug discovery and development. Photostability is important for functions like handling, packaging, and labeling of the compounds.[14]

Drug metabolism refers to the susceptibility of compounds to biotransformation which depends on the presence of groups in the molecule those are open to enzyme catalyzed transformation.[15] Metabolic stability studies are also very significant during the early stage of drug discovery to determine the drug metabolites and to detect the safety and efficacy of the drug leads before progress to the clinical trial.[16] The analytical tools like liquid chromatography-mass spectrometry (LC-MS) and RP-HPLC play prominent role in these processes.

The amount of actual drug present in a drug sample is termed as its potency, while the impurity in a drug substance, as defined by the ICH Guidelines, is any component of the drug sample that does not fall under the chemical entity defined as the drug substance, and thus affects the purity of the drug substance. The ICH Q3 Guideline classifies impurities as organic, residual solvents, and inorganic.[17] The Q3A and Q3B guidelines focus on the requirements to be classified as the organic impurities which are mainly of two types, process related impurities and degradation impurities. The organic impurities occurring during the manufacturing processes are called process related impurities which include intermediates, byproducts, transformation products, and interaction product, whereas those that may develop during storage of the drug substance/product are classified as degradation impurities. With the passage of time and continuous change in various affecting environmental factors such as temperature, humidity, and probable drug-excipients interactions, the potency of drug substances/product may vary (usually decrease), and eventually may lead to the generation of impurities, which may be toxic or cause undesirable health issues on patients.

The Q3C and Q3D Guidelines provide clarification of the requirements for residual solvents and metal impurities. Residual solvents are defined as organic volatile

chemicals which cannot be completely removed during the manufacturing of drug substances, its excipients, or drug products. Appropriate selection of the solvent for manufacturing a drug substance/product may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent used can be a critical parameter in the drug synthesis process. To ensure the quality of the product, content of the solvents should be tested and justified, and should be below the recommended limits of Class 1, 2 and 3 solvents. One of the powerful separation techniques used for the determination and quantification of residual solvents is gas chromatography which plays important role in the analysis of a pharmaceutical products. Analytical methods for resolving the residual solvents present in the drug substances and in the products are available in the United States Pharmacopoeia.[18] The elemental impurities present in drug products may come from a variety of sources such as the residual catalysts or may have been formed during the reaction through interactions with processing equipment or container/closure systems, or even just by being present in components of the drug product. Detection of elemental impurities is often done by the technique called atomic absorption spectroscopy. Analytical methods for determination of the elemental impurities present in the drug substances and product are also available in the United States Pharmacopoeia.[19]

The drug quality and safety is ensured by the application of drug manufacturing control measures that demand intense analytical and chemical support for all the stages of the manufacturing process, and that too at a very high level. Keeping the intended purpose of pharmaceutical drugs in mind, which is to cure or prevent the patient of disease, the drugs are expected to be free from impurities which might cause them to fall short of its purpose or harm the patients. For making sure that the drugs do their job efficiently and effectively, regulatory agencies recommend application of stability indicating methods (a validated analytical procedure that accurately and precisely measures the active ingredients in the drug product) for the thorough analysis of every samples keeping an eye on the content of drug substances, its impurities, and the degradation products generated during its shelf life. It is also highly recommended that the assay procedures carried out should be a stability indicating

method since these methods examine the drug product ultimately manufactured in the desired dosage form guaranteeing their safety, effectiveness and quality.

Due to the advanced level of techniques involved in it, RP-HPLC has emerged as a leading system used for drug component analysis during the various steps of drug development and manufacturing. Being a fast chromatographic technique of high flexibility such that samples of multiple varieties can be analyzed at once plus the falling of product analysis by RP-HPLC under the stringent regulations established by the U.S. Food and Drug Administration (FDA) have made the use of RP-HPLC for testing the quality of the products before they hit the global market, an obligation for all the pharmaceutical companies. The appropriate HPLC system operation and application depends on the operator's understanding of the principals of chromatographic process, the reasons for the choice of specific components in the chromatographic systems, and the procedure to be followed.

4.2 ANALYTICAL METHOD DEVELOPMENT BY RP-HPLC

Reversed Phase High-performance/High-Pressure Liquid Chromatography (RP-HPLC), is an analytical technique used in the field of analytical chemistry in pharmaceutical companies, research laboratories etc., to separate, to identify and to quantify the components in a mixture. RP-HPLC has a non-polar stationary phase (HPLC Column) and a moderately polar mobile phase which contains both aqueous and organic phase in a different ratio. With the help of a pump, the pressurized liquid solvent containing the sample mixture is passed through the HPLC column, where the components are separated by the separation mechanism discussed below and each component are identified in the detector. Figure 1 represents a schematic diagram a HPLC system.[23]

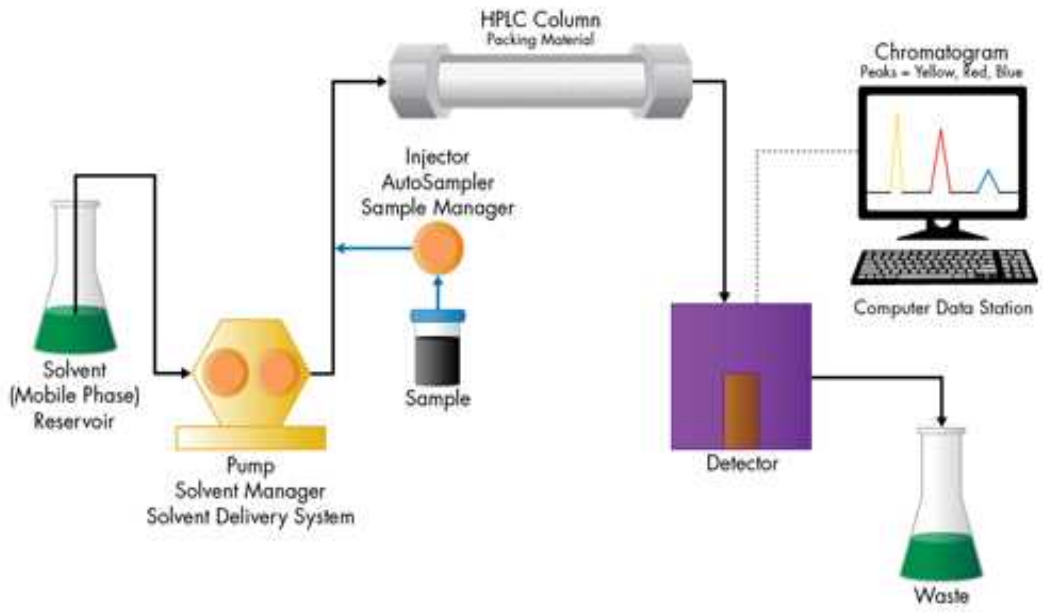


Figure 1: Schematic Diagram of an HPLC system.

In reverse phase chromatography, the separation mechanism relies on the hydrophobic and hydrophilic interaction of the components with the mobile phase and hydrophobic interaction of the components with the non-mobile hydrophobic ligands (C-8, C-18 etc.) in the stationary phase. Partition of the compound between the mobile phase and

the stationary phase depends on the rate of reversible adsorption and desorption with the degree of hydrophobicity of the compound to the hydrophobic stationary phase.

Method development is a process by which analytical procedures, pathways, and methods are developed for a given analyte to ensure its quantity or quality by various means. Analytical method development for pharmaceutical drug substances and products is a key factor in the field of pharmaceutical research and development. To develop a selective, precise, accurate, and robust RP-HPLC method, the following fundamental steps should be considered.

Step 1:
Physicochemical properties: pKa value, solubility and stability of the compound in buffer and organic solvent.

Step 2:
Preparation of test solution: percentage of organic solvent, buffer solution, shaking/sonication, and filtration process.

Step 3:
HPLC chromatographic conditions: HPLC column, mobile phase (pH of the buffer solution, percentage of organic and buffer solution), injection volume, isocratic/gradient elution mode, flow rate, column oven temperature, initial wavelength, and solution stability.

Step 4:
Method optimization (Preparation of test solution, concentration of test solution, modification of mobile phase (organic and buffer) ratio for better selectivity, selection of wavelength, evaluation of response factor, data integration and calculation procedure.

Step 5:
Perform the robustness test and modify the developed method (if needed).

Step 6:

Analytical Method validation.

In order to select the most appropriate chromatographic conditions for the HPLC method, one must understand the physiochemical properties. Solubility, pKa value, and stability of the drug substance help to select the most appropriate solvent to prepare the test solution and mobile phase. Distribution of species (ionized and unionized form) depends on the pH of the buffer solution with pKa value. Acidic compounds are completely ionized when the pH of the buffer solution is more than the pKa value (~pKa + 2). Hence, for acidic compounds pH (~pKa + 2) of the buffer solution should be more than the higher pKa value of the compound having multiple pKa values because at higher pH the compound will be completely ionized, soluble and the compound will also elute faster from the HPLC column. For basic compounds pH (~pKa - 2) of the buffer solution should be less than the lowest pKa value of the compound having multiple pKa values because at lower pH the compound will be completely ionized, soluble, and the compound will elute faster from the HPLC column. For neutral molecules any suitable pH can be selected.

Different types of reagents are often used to prepare the buffer solution (0.01 M to 0.5M). Acetate buffer is prepared from a mixture of acetic acid, and sodium acetate/ammonium acetate. Phosphate buffer is prepared from a mixture of phosphoric acid and monobasic/dibasic /tribasic potassium/sodium phosphate. A lower percentage (0.05% - 0.5%) of formic acid in aqueous phase is also used to prepare the mobile phase. 0.1 M Sodium hydroxide, hydrochloric acid, phosphoric acid and acetic acid, as appropriate, used to adjust the pH of the buffer solution. Acetonitrile and methanol are preferred as organic solvents because of their UV cut-off of 190 and 205 nm, respectively. At lower concentration (0.01% to 0.2 %) of Trifluoroacetic acid and Triethylamine are used in organic phase to reduce tailing peak by reducing the silanol activities in the case of acidic and basic compounds, respectively.

Endcapping refers to the replacement of accessible silanol groups in a bonded stationary phase with trimethylsilyl groups. Endcapping RP-HPLC column is most

often used because it cuts down the undesirable secondary interactions of ionic compounds and leads to good peak shape and selectivity. Efficiency, sensitivity, selectivity, and elution time of compounds also depend on the dimensions of the RP-HPLC column (internal diameter, length, and particle size). 4.6mm x 250mm, 5.0µm particle size and 4.6mm x 150mm, 5.0µm particle size are the most suitable dimensions for HPLC column. Endcapping C8-bonded and C18-bonded silica are used to prepare RP-HPLC column to separate a wide range of organic compounds and to separate the enantiomeric impurity chiral column.

Forced degradation:

Accelerating the degradation of drug/drug product by application of external degradation procedure is known as forced degradation. To perform the forced degradation, a test sample has to be exposed directly to the ambient/elevated temperature and moisture, which is called solid state exposure or the sample can be exposed into the liquid media and then refluxed/heated the test sample solution/mixture in a hot water bath for a specified time period. This method is called as liquid state exposure.

The other mode of degradation and conditions are given below:

Mode of Degradation	**Condition**
Neutral hydrolysis	Water
Acid degradation	≥ 0.1 M Hydrochloric acid.
Base degradation	≥ 0.1 M Sodium hydroxide.
Oxidation	≥ 30% Hydrogen Peroxide solution.
Thermal or Heat degradation	At a temperature between 60ºC and 105ºC for at least 24 hours.
Photo-degradation	1.2 million lux hours (which is the completion of one cycle in the photo-stability chamber).

Robustness:

The robustness of an analytical procedure is a measure of its capability to resist small changes in method parameters. It provides an indication of its reliability during normal usage and can be established by altering the following parameters:

a) Columns: Different brands of different manufacturers or different lots/serial number of same manufacturers.
b) pH of Buffer solution/Mobile phase: can be adjusted to within ± 0.05 to ± 0.5.
c) Ratio of components in mobile phase: can be adjusted to within ± 2% to ± 10%.
d) Wavelength: can be adjusted to within ± 1 nm to ± 3 nm.
e) Temperature: can be adjusted to ± 2º C to ± 10º C.
f) Flow rate of the mobile phase: can be adjusted to within ± 0.05 ml/min to ± 0.5ml/min.
g) Sample extraction time: can be adjusted within ± 1 to ± 10 min.

Data integration and calculation procedure:

The percentage purity of the principal compound and its related compound can be directly measured from the area of the peak [%Purity = (area of the compound/impurity)/Total area] which is called area normalization method. Relative Response Factor [RRF=slope (impurity) / slope (API)] value must be evaluated during the linearity study to calculate the percentage of related compound by area normalization method. The percentage purity of the principal compound and its related compound are usually measured by comparing the response of the test sample with the response of the pure reference standard; this is known as reference standard method.

Operational procedures to develop a HPLC method are as follows;

a) Prepare the test solution spiked with all known impurities having a concentration of 0.1 mg/mL and transfer in the HPLC vial.
b) Prepare 0.05 M acetate and phosphate buffer solutions
c) Select the C-18 endcapping HPLC Column with the dimensions of 4.6mm x 250mm, 5.0µm particle size.

d) Condition the column with one of the above buffer and organic phase (Acetonitrile) in the ratio of 50:50 for at least 30 min with a flow rate of 1 mL/min and column oven temperature 40º C.
e) Inject 10µl of the test solution and scan the sample from 200 nm to 400nm to get the lambda max of the compound.
f) Check the area and shape of the peak.
g) Adjust the injection volume and mobile phase ratio to get a good peak.
h) Replace the buffer solution and repeat the same procedure (iv to viii)
i) Replace the organic phase (methanol) and repeat the same procedure (iv to viii)
j) Check the system suitability and solution stability at least for 6.0 hours by comparing the area of the peak.
k) Select the most suitable buffer by comparing the above data.
l) Develop an isocratic/gradient elution mode with the suitable mobile phase to develop a suitable HPLC method. If necessary add trifluoroacetic acid/ triethylamine to reduce the peak tailing.
m) Perform forced degradation study to select the most suitable ratio/gradient program to confirm that there is no co-elution with any peak.
n) Confirm the capability of the method to resist small changes in method parameters by performing a robustness study to select the most suitable ratio/gradient program, flow rate, column oven temperature, sample preparation procedure.
o) Inject the test solution with the modified method to select the wavelength (principal compound).
p) Check the response and chose the test concentration.
q) Develop the data integration and calculation procedure.

4.3 ANALYTICAL METHOD VALIDATION BY RP-HPLC

Analytical method validation[20-22] is a documented evidence that proves that analytical method gives the same repeated outcomes or results for an analyte being tested. The important validation characteristics for different types of analytical procedures according to ICH guidelines are listed below. It should be noted that the selection of validation characteristics for a specific analytical method depends on the type, nature and application of the analytical procedure. Some of the examples of analytical methods are assay, related substance, residual solvents and dissolution test procedure. Analytical method validation is not required for routine pharmacopoeial test procedures like loss on drying, water content, residue on ignition etc.

Analytical method validation characteristics:

- System Suitability
- Filter Evaluation
- Specificity
- Forced Degradation
- Linearity
- Precision
    - System Precision
    - Method Precision or Repeatability
    - Intermediate or Ruggedness
    - Reproducibility
- Accuracy/Recovery
- Limit of Detection
- Limit of Quantitation
- Range
- Solution Stability
- Comparative Study

- **System suitability testing**

  System suitability testing is the method which ensures that both system and analytical method are appropriate for analysis and validity of the data provided from that instrument. Some commonly used system suitability parameters to check the performance of the HPLC system and the analytical method are as follows:

  - Percentage relative standard deviation (%RSD) of area and retention time of the replicate measurements of chromatographic peak are essential criteria for system suitability testing. Based on the type of analytical method, the acceptance limit varies from 2.0% to 15.0%.
  - Resolution/Peak to valley ratio between two adjacent peaks is also one of the most important criteria for system suitability testing for the related substance test method. The minimum limit for resolution is not less than 1.5 and for peak to valley ratio is not less than 1.2.
  - The tailing factor is a measure of peak tailing and for a good peak shape the tailing factor should be close to 1.0.
  - Theoretical plate number is a marker of column efficiency and the minimum limit is not less than 2000 but it may vary based on the analytical method and HPLC column.

- **Filter Evaluation Study**

  Filter evaluation study is carried out to evaluate the most suitable filter for the purpose of the filtration of the test solution. For this, test solution is filtered through different types of filter papers and results are evaluated in comparison with the results of unfiltered solution.

- **Specificity**

  Specificity is the ability to assess an analyte distinctively in the presence of unwanted compounds like impurities, degradation of products, and matrix components other than the analyte itself. Specificity of the method can be evaluated by carrying out following tests:

- Identification tests:

  Used to distinguish between closely related compounds, identification test can be achieved by comparing the results between sample and reference standard. For chromatographic method if the ratio of the retention time of standard and sample is 0.95 to 1.05 then they are consider as closely related compounds.

- Placebo interference:

  Placebo interference study is carried out by spiking pure drug substance with appropriate levels of excipients and by demonstrating that the assay result remains unaffected by the presence of these excipients and is compared with the unspiked sample results.

- Impurities interference:

  Impurities interference study is carried out by spiking impurities at specification level with appropriate levels of pure drug substance/excipients and by demonstrating that the assay result remains unaffected by the presence of these impurities and the resolution between two adjacent compounds should be minimum of 1.5.

- **Forced degradation study**

  The forced degradation study is discussed in method development section above. This study should be performed again during method validation to conform that there is no co-elution of any degradants with the principal peak.

- **Linearity**

  Linearity of a method is the ability of the analytical procedure to show linear relationship in response to the change in concentration of analyte. For the establishment of linearity, a minimum of five concentrations is recommended. Regression analysis is used to evaluate a linear relationship between test results which is usually evaluated across the range of the analytical procedure. The data obtained from analysis of the solutions prepared at a range of different concentration levels is usually investigated by plotting on a graph. For relationship to exist correlation

coefficient (R^2), derived from regression analysis (y = mx + c), should be more than 0.99.

- **Precision**

Precision of an analytical procedure is the degree of scatterings of the measurements obtained for a series of data. The test samples should be prepared from the same mixture or same lot. A minimum of six results should be measured. Precision is often expressed as the standard deviation (co-efficient of variation) of the measured results. The standard deviation and the relative standard deviation can be reported for each type of precision and %RSD can vary from 1.0% to 15.0% based on the types of analytical method.

The following levels of precision are recommended:

- System Precision

System Precision is usually demonstrated by repeated injection of the standard solution. A minimum of three determinations at 100% of the test concentration is recommended.

- Repeatability (Intra-assay precision/Method precision):

Repeatability refers to the precision for the data obtained under the same operating conditions over a short period of time. It is recommended that a minimum of six replicate sample determinations should be made.

- Intermediate Precision:

Intermediate precision, also known as ruggedness, is carried out to ensure that the method will provide same results when done in similar on different day(s) within the same laboratory. Generally the experiment is repeated on the same sample by a different analyst, on a different day, using different equipment.

- Reproducibility:

  Reproducibility is assessed by means of an inter-laboratory trial. After intermediate precision has been carried out reproducibility is done for the same experiment is repeated on the same sample by a different analyst, on a different day, using different equipment in a different laboratory.

- **Accuracy**

  The accuracy of an analytical procedure is the degree of closeness/trueness of the value obtained through the experiment conducted to that of the true value. The accuracy of an analytical method is established through the range of an analytical method and it is determined by measuring the percentage recovery of the drug substance/impurities from the samples spiked with known amount of drug substance/impurities and with the excipients as appropriate. A minimum of nine determinations for three concentration levels covering the range should be measured. %Recovery value should be 98.0% to 102.0% for assay, 95.0% to 105.0% for dissolution and 85.0% to 115.0% for impurity test procedure.

- **Range**

  It is the highest and lowest concentration of the analytical method which has been proved to be linear, accurate and precise.

- **Limit of detection and Limit of quantitation**

  Limit of detection is the quantity in which the analyte can be detected. Limit quantitation is the lowest concentration that can be used to quantify the analyte. Limit of detection and Limit of quantitation can be established by the following procedures:

- Signal-to-Noise Approach:

  Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit and 10:1 for estimating the quantitation limit.

- Standard Deviation of the Response and the Slope:

  A specific calibration curve should be studied using samples containing an analyte in the range of detection limit and quantitation limit. The residual standard deviation of a regression line can be used as the standard deviation.

  The detection limit may be expressed as:

  Detection limit = 3.3 σ /s

  Quantitation limit = 10 σ /s

  Where,

  σ is the standard deviation of the response (estimated from the calibration curve of the analyte) and s is the slope of the calibration curve.

- **Solution Stability Study**

  Stability of sample solution at room temperature or specified condition can be determined by measuring the response of the analyte after specific intervals e.g. 3, 6, 12, 18 and 24 hours. Similarly stability of mobile phase can be determined by using same mobile phase for one week by checking the system suitability.

- **Comparative Study:**

  It is the study performed to verify the results and the accuracy obtained by different methods or same method. Results obtained by any proposed analytical method can also be verified by a comparative study with the results obtained by another validated analytical method. For instance, methods developed to determine the physicochemical properties like ionization constant and lipophilicity should be validated by comparative study with another method.

5.0 CHAPTER-II

Title:

Development and Validation of RP-HPLC methods to determine purity, solubility, stability and pKa of a novel antimalarial drug lead, Cyclen Bisquinoline.

5.1 INTRODUCTION

Amongst the numerous life threatening health problems world is facing today, malaria has been very prominent especially in developing countries of Southeast Asia, South America, and South Saharan Africa.[1] The 4-aminoquinoline based drugs, for instance, chloroquine that has been profoundly used as an anti-malarial agent, has now been proven to be less effective towards its resistant strains.[2] Even with potent curative effect, artemisinin and its derivatives also have issues like thermal instability[3,4] and high cost[5] which make its access in developing countries very limited. Addressing to all these limitations, taking an advantage of the similarity in structure with heme, and ability to bind with metal complexes, tetraazamacrocycles (e.g., Cyclen) based antimalarial drugs are being discovered. Cyclen based anti-malarial drugs showed potent *in vitro* and *in vivo* anti-malarial activity. The drug lead, Cyclen Bisquinoline (CNBQ) (Figure 2) with IC_{50}s of 7.5 and 19.2 nM against chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparum*, respectively, was proven to have the most potent anti-malarial activity.[6]

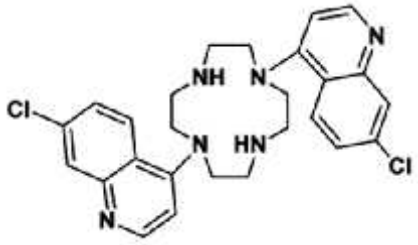


Figure 2. 4,10-bis (7-chloroquinoline)-1,4,7,10-tetraazacyclododecane (Cyclen Bisquinoline, CNBQ).

This lead structure (CNBQ) and the related compounds fulfilled important criteria for promising new antimalarial drug leads and warrant further study: 1) active against chloroquine-resistant as well as multidrug resistant isolates of *P. falciparum*, 2) simple pharmacophore structure that will afford low cost manufacturing, 3) orally effective in non-clinical malaria model, 4) potential to be developed as a single dose antimalarial drug, 5) long half-life and metabolic stability against human liver microsomes as well as CYP2C8, enzymes responsible for chloroquine metabolism.[6,7]

Early evaluation of compounds with drug-like properties will be helpful for speculation and recognition of potential challenges during the process of drug discovery and development. Evaluation of physicochemical properties not only saves time but also makes the process economic. These evaluations have significantly decreased the attrition rate in drug discovery and development due to factors like suboptimal physiochemical parameters, pharmacokinetics, and bioavailability. Compounds with suitable biopharmaceutical perspective and optimal physicochemical parameter such as solubility, lipophilicity, ionization constant and stability should be selected for discovery and development.[8] As physicochemical properties relates to the absorption, distribution, metabolism, and excretion (ADME) process of drugs *in vitro* and *in vivo*, these characterizations will be helpful in selection, optimization, and development of potential drug-like compounds.[9]

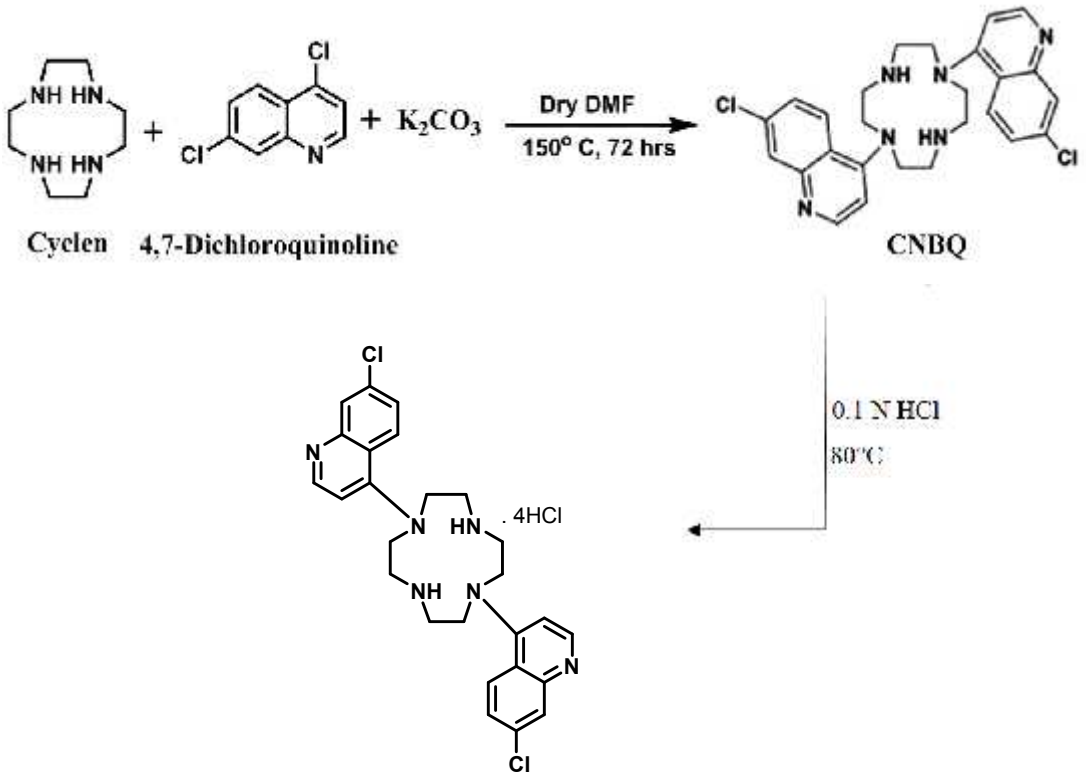
RP-HPLC method development and validation to determine the lipophilicity (log *P*), have already been reported for the drug lead CNBQ.[10] This chapter reports the detail of drug characterizations, and development and validation of RP-HPLC methods to determine the purity, solubility, stability and pKa of the antimalarial drug lead CNBQ.

5.2 DRUG LEAD SYNTHESIS AND CHARACTERIZATION:

Cyclen Bisquinoline, CNBQ is chemically 4,10-bis (7-chloroquinoline)-1,4,7,10-tetraazacyclododecane (Figure 2). Its molecular formula is $C_{25}H_{28}Cl_2N_6$ and molecular weight is 495.0. The compound has four different crystalline polymorphs. The Free base (FB) is a white polymorphic crystalline powder; the polymorphs melt at 166°, 178°, 195°, and 234°C, respectively. The salt is off-white powder that showed a broad endotherm in differential scanning calorimeter (DSC) analysis suggesting it to be amorphous.

FB of the CNBQ was synthesized in the laboratory according to the literature method (Scheme 1).6,11 All the reactions were performed using anhydrous solvents under inert conditions and the products obtained were monitored using TLC, HPLC, LC-MS and NMR that resulted to be relatively pure and stable. The FB was recrystallized using ethanol and acetonitrile. Hydrochloride salt of the FB was prepared treating with 0.1 N hydrochloric acid at 80°C and was crystallized from methanol : water (50:50). The percentage elements of hydrochloride salts of CNBQ obtained by PerkinElmer (Series II) Autosampler Carousel on the 2400 CHN Elemental Analyser are C, 47.97; H, 5.35; N, 12.96, which are within the limit of calculated elemental analysis results C, 48.70±0.4; H, 5.03±0.4; N, 13.11±0.4.

Infrared spectrum of the compound was obtained using Thermo Scientific Nicolet 380 FT-IR Spectrometer. Hydrochloride salt of the CNBQ was confirmed by two ways. One by its improvement of solubility in water (370-fold) and other by the absence of peak at 3294.82 in FT-IR spectrum as shown in figure 3. ^{1}H NMR spectrum and Mass spectrum are shown in figure 4. In the previous studies, mass data and NMR data of CNBQ were published.[11]



CNBQ HYDROCHLORIDE *(*MW: 641.284)

Scheme 1: Representing the synthesis of CNBQ and its hydrochloride salt.

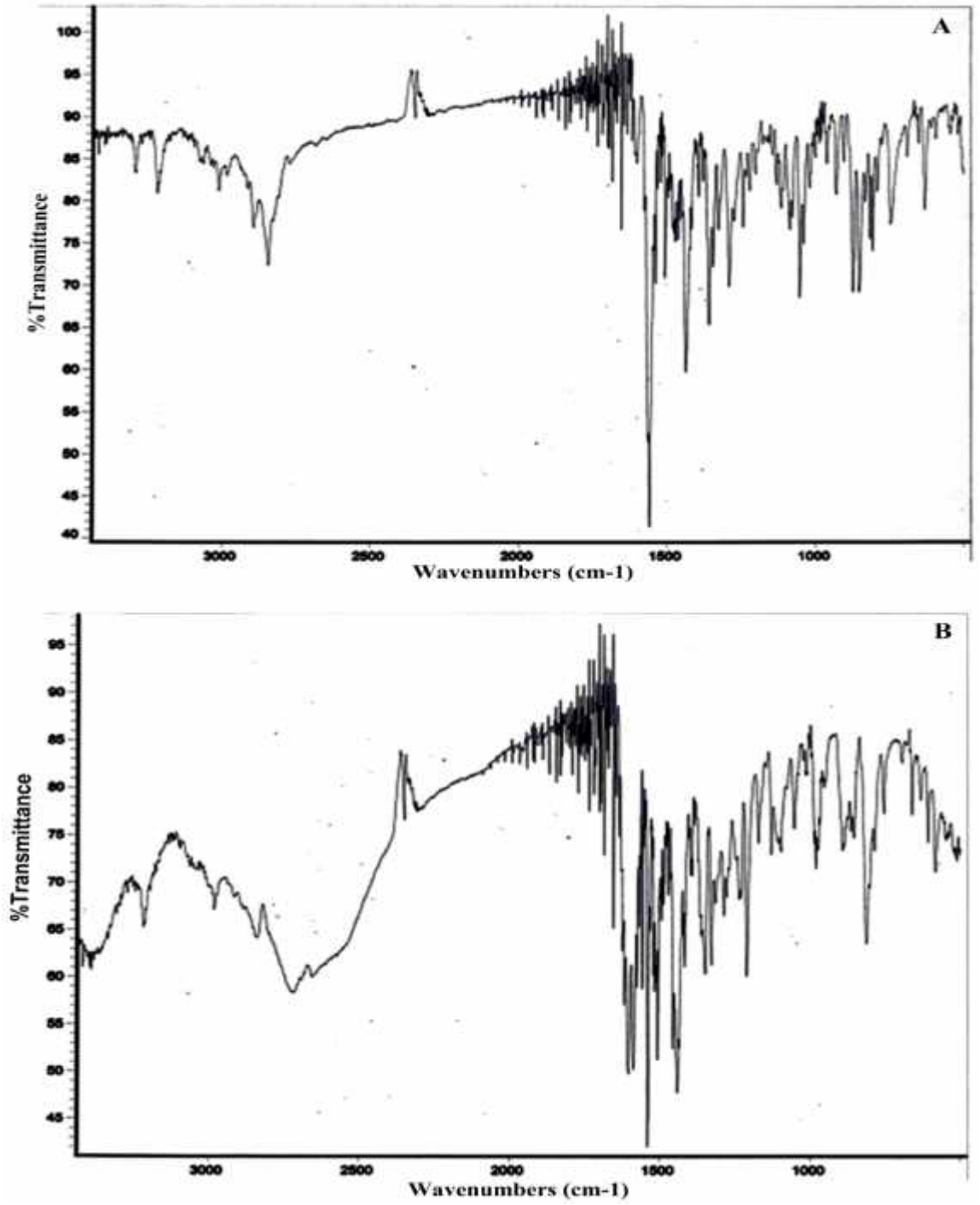


Figure 3. FT-IR spectrums of; A: CNBQ FB and B: its hydrochloride salt.

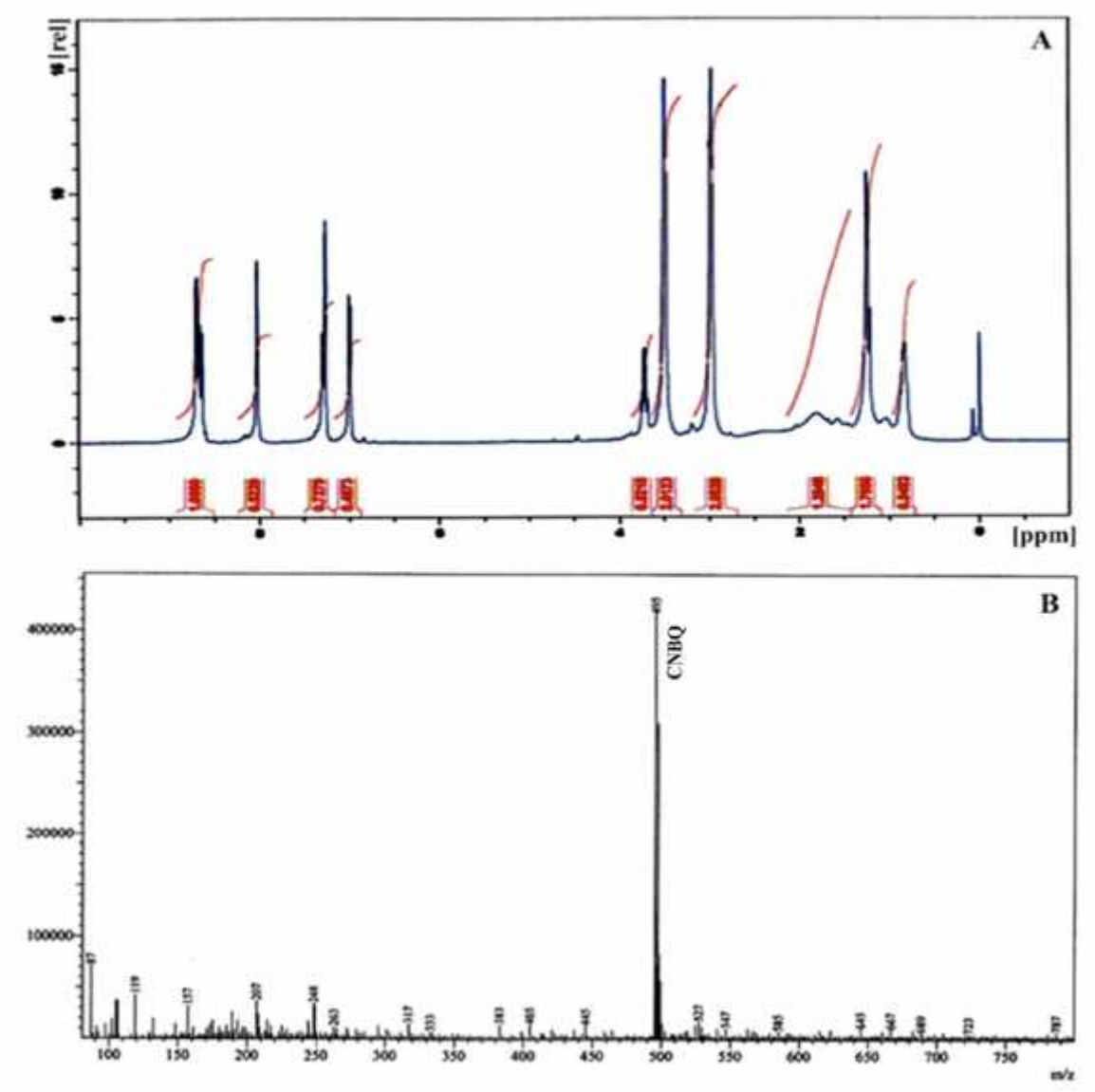


Figure 4. A: ^{1}H NMR spectrum and B: Mass spectrum of CNBQ FB.

Occurrence of solid-state phase transformation is very prominent during drug and its formulations development due to processing or drug-excipient(s) interaction.[12-14] Determination and quantification of phase transition and conformational changes hold significant value in drug discovery and development. This phase transformation may have a huge affect on factors like dissolution, bioavailability, and stability of the drug. The energy of phase transition, conformational changes, and melting point for both FB and salt of CNBQ was determined using differential scanning calorimeter (DSCQ2000) from TA instruments. For the DSC analysis, 1-2 mg of drug sample was

taken in a tzero pan-lid and, sealed using a compressor. Thermal events were quantified using universal analysis 2000 version 3.9A software. The compounds were either heated or subjected to heat-cool-heat cycle. In the first cycle (cycle 1), the compounds were heated at a constant rate of 5^{o}C/ min above their melting points but below their degradation temperatures and kept at this condition for one minute. In the second cycle (cycle 2) the compounds were cooled at 5^{o}C/min and reheated again at 5^{o}C/min in the third cycle (Cycle 3) (Table 1). The thermal events such as glass to rubber transition, recrystallization and melting were detected and quantified.

Table 1: Method for DSC analysis

Sample Purge flow	50 ml/min
Flange Temperature	25°C
Heater Temperature	25°C
Heater Temperature	25°C, Isothermal for 1 min
Ramp (Cycle 1)	5°C /min to 250°C
Ramp (Cycle 2)	5°C /min to 25°C
Ramp (Cycle 3)	5°C /min to 250°C

The FB (crystallized from Acetonitrile) was heated at 5^{o}C and also subjected to heat cool heat cycle. In both experiments a sharp melting endotherm was observed. However, in the former experiment, the melting endotherm showed it appearance at 235.88^{o}C with an enthalpy of fusion of 90.09 J/g (Figure 5). This suggests that the compound was crystalline in nature with only one physical form. However, in the later experiment, a melting endotherm was observed at 234.90^{o}C (enthalpy of fusion = 81.85j/g) (cycle 1) (Figure 6). The compound, when cooled after keeping it at 250^{o}C for one minute, a broad exotherm appeared at 127.12^{o}C with an enthalpy of fusion of 39.58 J/g, suggesting that the compound recrystallized on cooling (cycle 2). The compound was then heated again (cycle 3) and a melting endotherm appeared at 230.72^{o}C with an enthalpy of fusion of 70.05J/g. The results were in congruence with the former experiment, confirming that the compound is crystalline in nature with only

one physical form. The shift in melting endotherm by 4°C from the cycle 1 is due to presence of the both crystalline and amorphous form the compound. Since, melting point is a colligative property; presence of amorphous form could have caused a shift in melting endotherm. However, in both cases since a single melting endotherm is observed, it clearly suggests that the compound crystallized form acetonitrile existed only in one physical form.

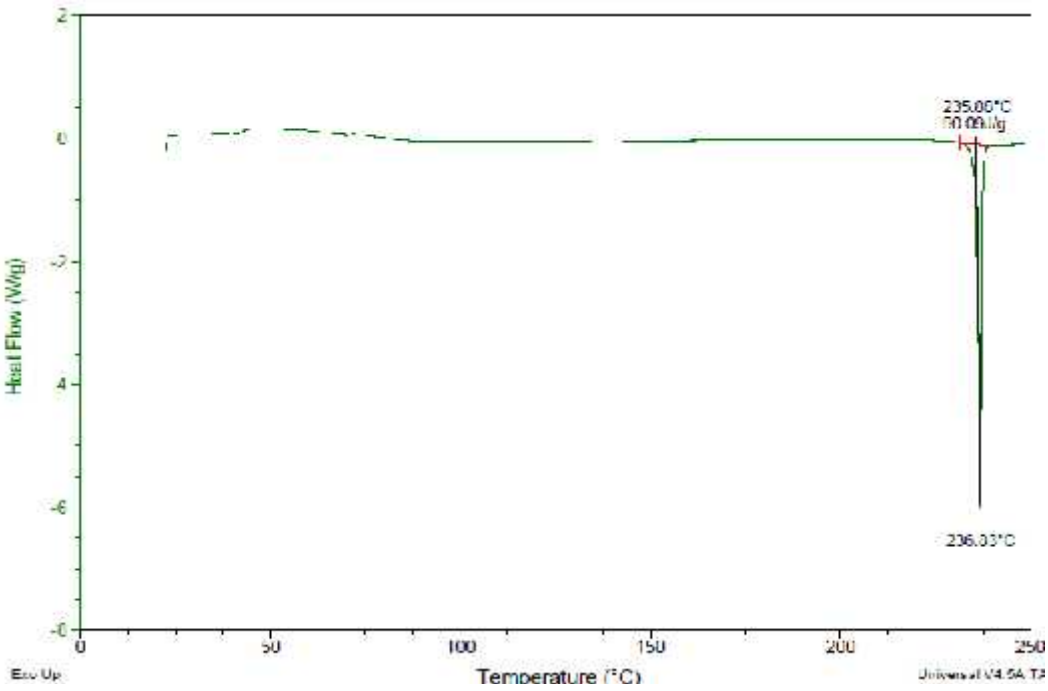


Figure 5. DSC Thermograms; Heat cycle for Free Base of CNBQ (Single polymorph).

Figure 7, shows the DSC thermogram of the compound crystallized from ethanol. In the first cycle, three sharp melting endotherms were observed at 164.66°C, 175.79°C and 193.50°C, with an enthalphy fusion of 31.33 J/g, 28.36 J/g and 74.63 J/g respectively, suggesting three different crystalline polymorphic forms of the drugs. In cycle 2, a small exotherm at 116.49°C was observed, however, in cycle 3 only one melting endotherm (relatively broad peak compared in cycle 1) was observed at 229.92°C with and enthalpy of fusion of 55.86J/g. The melting endotherm in cycle 3 was found to be closer to the one observed in cycle 3 in figure 6. This suggests that the

three crystalline forms seen in cycle-1, amorphousized on melting. However, some portion of the amorphous form recrystallized in cycle 2 and in cycle 3 the mixture of amorphous and crystalline form when heated, melted at 229^{o}C. It also suggests that the polymorph obtained at 229^{o}C is the stable polymorph. Even though three different polymorphs were identified in cycle 1, but it recrystallized to the most stable form on cooling.

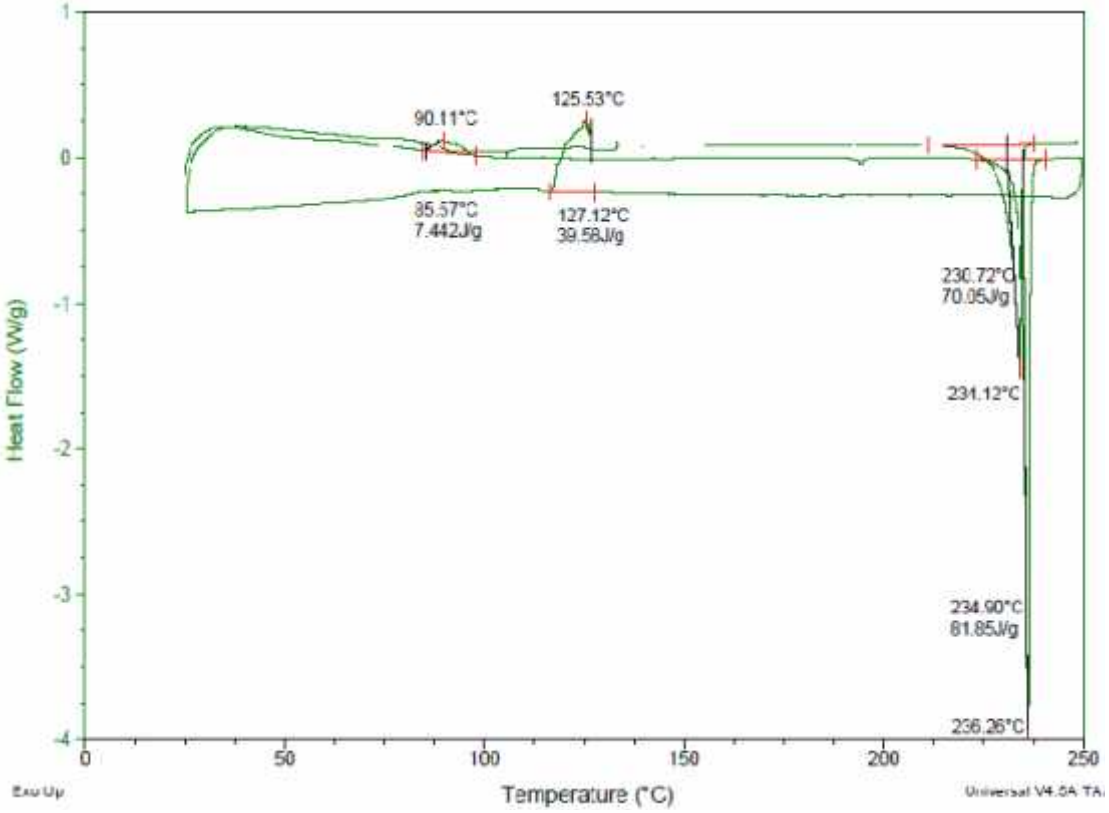


Figure 6. DSC Thermograms; Heat-Cool-Heat cycle for Free Base of CNBQ (Single polymorph).

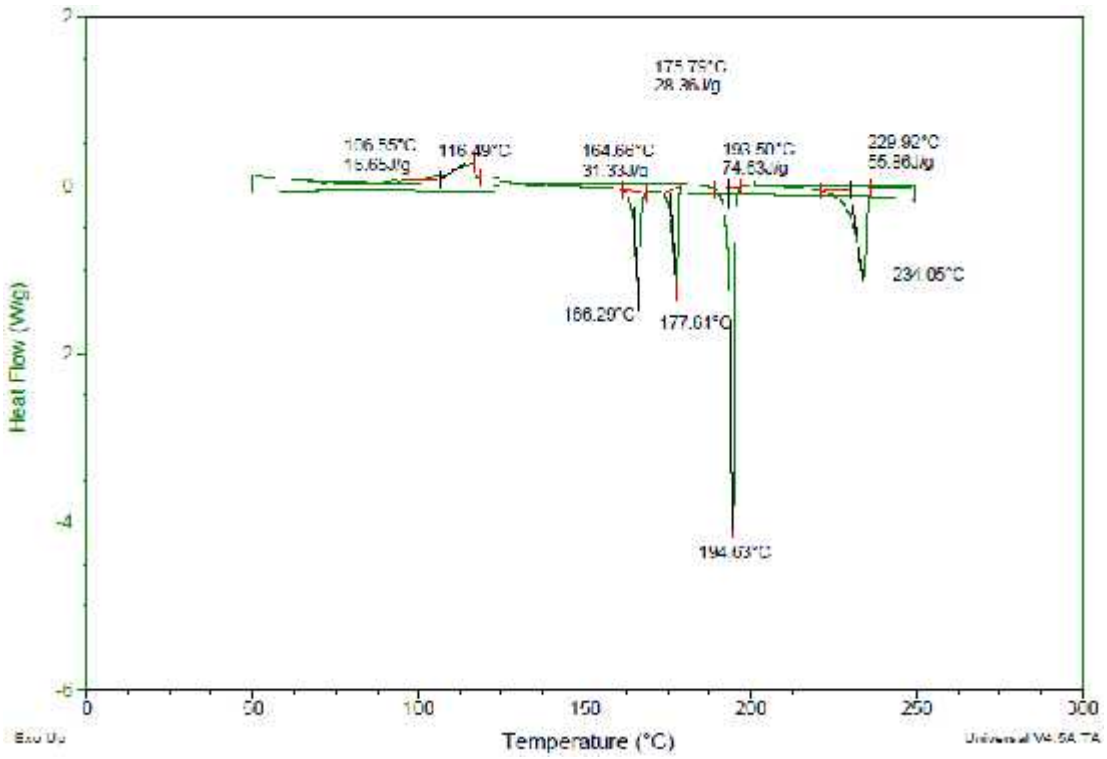


Figure 7. DSC Thermograms; Heat-Cool-Heat cycle for FB of CNBQ (Multiple polymorphs).

In Figure 8, the hydrochloride salt of the FB was heated up to 300^{o}C. A broad endotherm was observed at 100^{o}C. This suggests the loss of residual solvent from the compound evaporated on heating. A broad endotherm was also observed at 260^{o}C. The DSC results indicate that the salt form is amorphous in nature due to the absence of any crystalline melting endotherm. The compound started to degrade when heated above 270^{o}C.

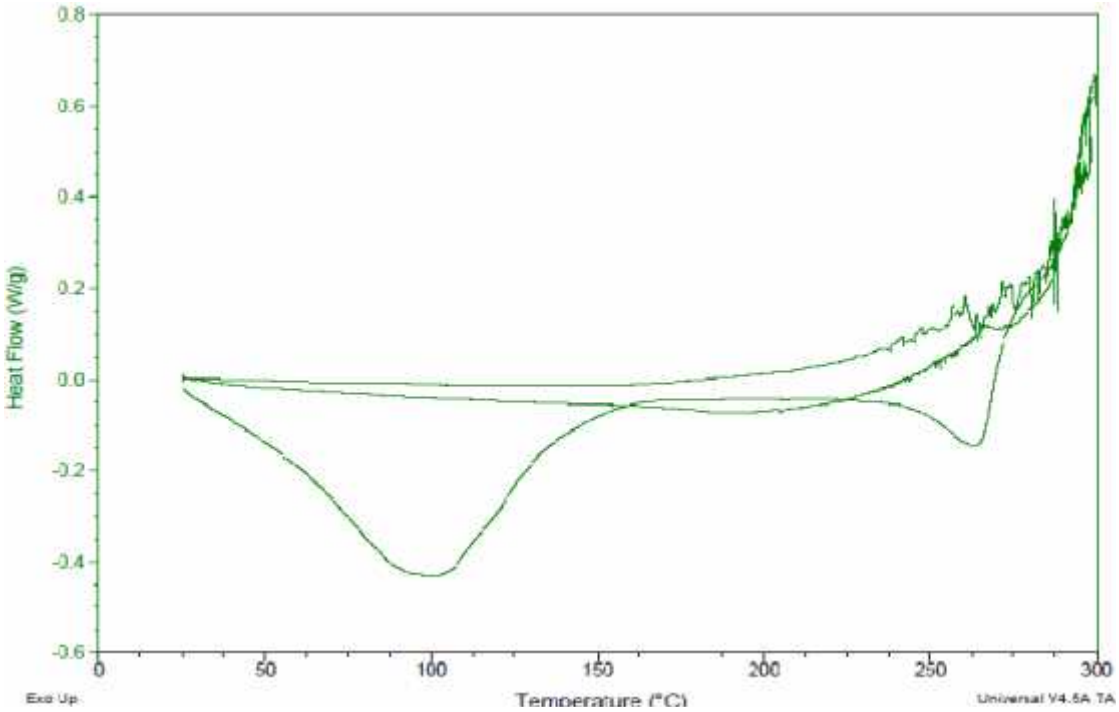


Figure 8. DSC Thermograms; Heat-Cool-Heat cycle for Hydrochloride Salt of CNBQ.

The melting point was determined by using Digimelt MPA 160 from SRS. For that drug substance was packed in the capillary tubes and placed into the Digimelt oven. Then the melting point analysis was carried out within the temperature range of 25°C - 260°C at the rate of 5°C/min. It was observed that FB of CNBQ melted at 234°C to 236°C whereas hydrochloric salt of the FB did not melt until 250°C.

5.3 SOLUBILITY STUDY

5.3.1 Abstract

The purpose of this study was to develop and validate a RP-HPLC method to evaluate the solubility of a novel antimalarial drug lead, CNBQ (free base; FB) and its HCl salt. Chromatographic separation of drug lead CNBQ, was successfully achieved on a C-18 column (4.6mm x 250mm, 5.0µm) in an isocratic separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with Phosphoric Acid in the ratio of (70:30, v/v). The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325 nm. Equilibrium solubility and stability of both FB and its salt were carried out in different mediums and samples were analyzed using RP-HPLC. The equilibrium solubility order of the FB is: DMSO > ethanol > pH 1 > isopropyl alcohol > tween 80 > propylene glycol > PEG 400 > water > pH 4.5 > pH 7.4 > pH 9.0 > pH 12.0; and that of salt is: water > propylene glycol > DMSO > ethanol > PEG 400. It is highly hydrophobic, however, salt formation improved its water solubility by approximately 370-fold.

5.3.2 Introduction

Solubility of the drug is referred as a maximum dissolved concentration in a specific condition. Besides properties of the drug molecules and solvent, factors like polarity, ionization potential, size, form, and pH of the drug has a huge effect on the solubility.[8] Thermodynamically stable crystalline form will be more stable and hence less soluble whereas amorphous form will be less stable, more soluble, and prone to crystallization and degradation.[15] Thus, solubility is an important factor to be evaluated in the process of drug discovery and development. It can create obstacles during the development process.[9]

Solubility profile of the compound in different media are the primary requirement that should be evaluated before any drug development from its discovery stage. In this experiment all the solvents and media were selected to determine the solubility aiming

to develop the pharmaceutical dosage form. They assist in evaluating appropriate dosage form that would be required to resolve any complications during the process and can be implemented in the modern quality by design (QbD) approaches for better progress of the drug development. Methods like titration, UV-Spectrophotometry, and RP-HPLC can be applied for the determination of solubility. However, RP-HPLC technique is a proficient way to determine the solubility of a drug substance in the early stage of drug development because of its high detection, accuracy and precision level and requirement of lower amount of sample.

5.3.3 Materials and Methods

5.3.3.1 Materials:

Ethanol, DMSO, Isopropyl Alcohol, Tween 80, Propylene glycol, and polyethylene glycol 400 (PEG 400), solvents used for solubility study were purchased from Fisher Scientific. While methanol, sodium hydroxide, hydrochloric acid, triethylamine, sodium phosphate dibasic anhydrous and phosphoric acid were purchased from Fisher Scientific. HPLC/LC-MS grade water was bought from Fisher Scientific, and the deionized water used was further purified by filtration and degassing. The drug lead mentioned in the figure 2 was synthesized in the laboratory.

5.3.3.2 Experimental Methods

Solubility study for free base was carried out using various solvents (DMSO, ethanol, isopropyl alcohol, tween 80, propylene glycol, PEG 400, water, pH 1, pH 4.5, pH 7.4, pH 9.0, pH 12.0). Salt solubility was determined in solvents like water, propylene glycol, DMSO, ethanol, and PEG 400. Substrate was added to both FB and salt solvents till saturation and were sonicated for 5 minutes and kept in shaker for 24 hours at 25°C. To quantify the saturation level of the drug in the above mediums, a standard solution of FB (0.0067 mg/ml) and salt (0.0067 mg/ml) were prepared using diluents and analyzed by RP-HPLC.

Chromatographic conditions:

Chromatographic separation of CNBQ was obtained from Waters X-Bridge C-18 column (4.6mm x 250mm, 5.0µm particle size, part no. 186003117) purchased from Waters Corporation. An isocratic separation mode with mobile phase consisting of 70% of 0.1% triethylamine in methanol and 30% of 0.02 M dibasic sodium phosphate (anhydrous) was used and pH 3.5 was adjusted with phosphoric Acid. Mixture of acetonitrile and water (50:50) were used as diluents. The instrumental set up included the flow rate which was maintained at 1.0 ml/min, and temperature of the column oven at 40°C. The injection volume was set to 5 µl and the run time for 10 minutes. Effluent was detected at 325 nm.

5.3.4 Analytical Method Validation Report

To validate the test method, different analytical performance parameters such as system suitability, linearity, accuracy, specificity, precision, solution stability and robustness were determined according to ICH issued analytical method validation guidelines Q2 (R1).[16]

5.3.4.1 System Suitability:

To assess system suitability of the proposed method, repeatability, theoretical plates, tailing factor, and retention time of six replicate injections of standards were measured. Percentage relative standard deviation (%RSD), tailing factor and theoretical plate values were calculated in each case. The results (Mean ± %RSD of six replicates) of the chromatographic parameters were plotted in table 2 indicating the good performance of the system.

Table 2: Chromatographic characteristics of system suitability for CNBQ (FB and hydrochloride salt.

Parameter	Value (Mean ± %RSD)	
	FB	**Hydrochloride Salt**
Peak area	460.131± 0.4	379.012 ± 0.3
Tailing factor	1.2 ± 0.1	
Theoretical plate	2132.5 ± 0.5	

5.3.4.2 Specificity:

The specificity of the developed method was determined by blank analysis and checking peak purity of the test solution. From this study, it is observed that each component gave response separately with respect to retention time and passed peak purity. No interference due to blank was observed in the chromatogram for blank solution (Figure 9). Therefore, the method is considered to be specific.

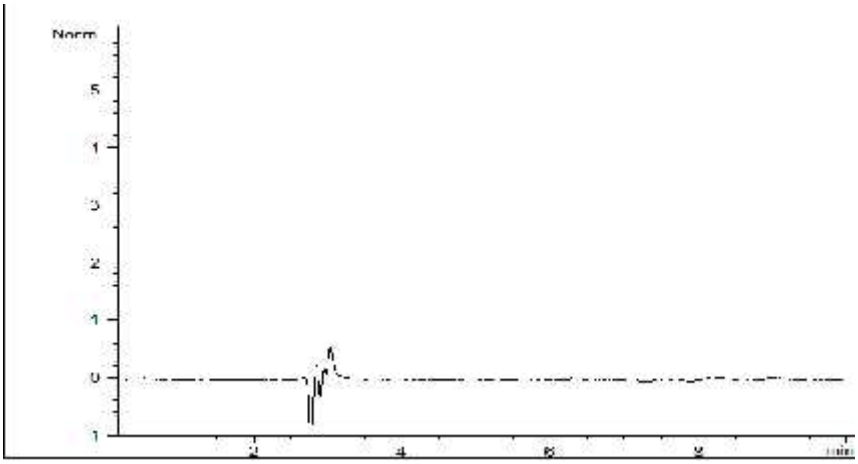


Figure 9. HPLC Chromatograms obtained in solubility studies; Blank (acetonitrile and water (50:50).

5.3.4.3 Linearity

The linearity was evaluated by analyzing different concentration of standard solutions of FB and its salt over the concentration range of 0.002 mg/mL to 1.072 mg/mL by linear regression analysis, which was then evaluated by the least-square

regression analysis. In both cases the correlation coefficient were within the limit of not less than 0.99 (FB: y = 7033.1x + 3.9223, R^2 = 0.9998 and Salt: y = y = 5765.3x + 8.7098, R^2 = 0.9995). Thus the method is considered to be linear.

5.3.4.4 Precision

The repeatability (intra-day precision) of the method was assessed by performing replicate measurements (n=6) of both FB and salt of CNBQ and the intermediate precision (inter-day precision) of the method was assessed by performing replicate measurements (n=6) of both FB and salt of CNBQ form the same batch by different analyst on different dates. The results were expressed as %RSD of the measurements. %RSD of the repeatability were found 0.59% and 0.41% and %RSD of intermediate precision were found 1.02% and 0.91%, respectively. All the results were found within the acceptable limit of not more that 2.0%. Thus, the method is considered to be precise.

5.3.4.5 Accuracy

Recovery of the proposed method was conducted at 50%, 100%, and 200% of nominal test concentrations and the percent recoveries were calculated. All the results were found within the acceptable limit of 98.0% to 102%.

Table 3: Accuracy studies of FB and salt of CNBQ.

Sample Name	%Recovery	
	FB	**Salt**
50% Sample 1	99.2	100.9
50% Sample 2	100.5	98.3
50% Sample 3	98.7	101.4
100% Sample 1	99.4	100.2
100% Sample 2	99.1	99.5
100% Sample 3	101.2	99.7
500% Sample 1	101.1	101.4
500% Sample 2	99.8	101.6
500% Sample 3	98.9	98.6
Mean	99.8	100.2
Standard Deviation	0.95	1.24
%RSD	0.95	1.23

5.3.4.6 Sample Solution Stability

To check the solution stability, the test sample solution was kept in room temperature and light for two days and measured the peak area of the analyte after specific intervals of 3, 6, 12, 18, 24, 36 and 48 hours and the percent difference between initial results were calculated. The test sample solution of both FB and salt of CNBQ were found to be stable up to 48 hours at room temperature because the percent differences between initial results were 0.72% and 0.51% at 48 hours, respectively.

5.3.4.7 Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. To determine the robustness of the current method, pH of the buffer solution was assessed at 3.4 and 3.6 instead of 3.5. The effect of flow rate was studied at 0.9 and 1.1 $mLmin^{-1}$ instead of 1.0 $mLmin^{-1}$. The effect of column temperature was studied at 35° and 45°C and mobile phase composition was assessed at Buffer :

Organic = 65 : 35 (v/v) and Buffer : Organic = 75 : 25 (v/v). The system suitability parameters under these conditions were calculated in all cases and found within the acceptable limit (%RSD of peak area NMT 2.0%, Tailing factor NMT 2.0, and Theoretical plate NMT 2000).

5.3.5 Results and Discussion

Under the provided experimental conditions carried out on various medium, it was observed that the equilibrium solubility order of the FB is: DMSO > ethanol > pH 1 > isopropyl alcohol > tween 80 > propylene glycol > PEG 400 > water > pH 4.5 > pH 7.4 > pH 9.0 > pH 12.0; and that of CNBQ salt is: water > propylene glycol > DMSO > ethanol > PEG 400. It was previously shown that CNBQ is highly lipophilic with a log *P* value of 5.14, signifying very low water solubility and high lipid solubility.[10] The observed solubility data of the FB is in consistent with its log *P* value. It was also shown that the compound is a weak base with pKa values of 5.9, 6.6 and 8.7.[17] As expected of any base, the salt formation improved its water solubility considerably. According to the solubility ratios of salt and FB, the salt formation improved its water solubility by approximately 370-fold (Table 4). The results showing the solubility of FB and its hydrochloride salt in different medium and pharmaceutical excipients are shown in table 4. For instance, HPLC chromatograms obtained during solubility studies of FB and its salt of CNBQ in water are shown in figure 10. To quantify the saturation level of the drug in water, a known concentration of standard solution of FB (Figure 10-A, 0.0067 mg/ml) and salt (Figure 10-C, 0.0067 mg/ml) were prepared using acetonitrile and water (50:50) as diluent and analyzed by RP-HPLC. AUC of the both standard solution were used to calculate the unknown amount of drug substance dissolved at their saturation level using their AUC (Figure 10-B for FB; saturation level 0.03 mg/ml and figure 10-D for salt; saturation level 8.581 mg/ml).

Table 4: Solubility of FB and hydrochloride salt of CNBQ in different solvents and excipients.

Solubility (mg/ml) of the FB and Salt in Different Media				**Solubility of FB in different Buffer Solutions**	
Media	**FB**	**HCl salt**	**Solubility Ratio (Salt/FB)**	**pH of the Buffer**	**Solubility in mg/ml**
.	0.183	0.234	1.7	pH 1	0.555
PG	0.204	1.080	6.9	pH 4.5	0.012
Water	0.030	8.581	370.2	pH 6.8	0.003
Ethanol	0.868	0.455	0.7	pH 7.4	0.008
DMSO	3.323	0.887	0.4	pH 9.0	0.008
Tween 80 (1%)	0.274	---	---	pH 12	0.000
Isopropyl alcohol	0.359	---	---	---	---

To increase the water solubility and thus dissolution in the stomach, basic drugs are generally administered in their hydrochloride salt forms. As the experimental outcome shows that the aqueous solubility of CNBQ has been increased by approximately 370-folds in its hydrochloride salts form, it will be very effective for future development as per FDA requirments[18] and formulation efforts of the compound. Further solubility studies on excipients and solvents were carried out to expedite the dosage form design for clinical studies of the CNBQ.

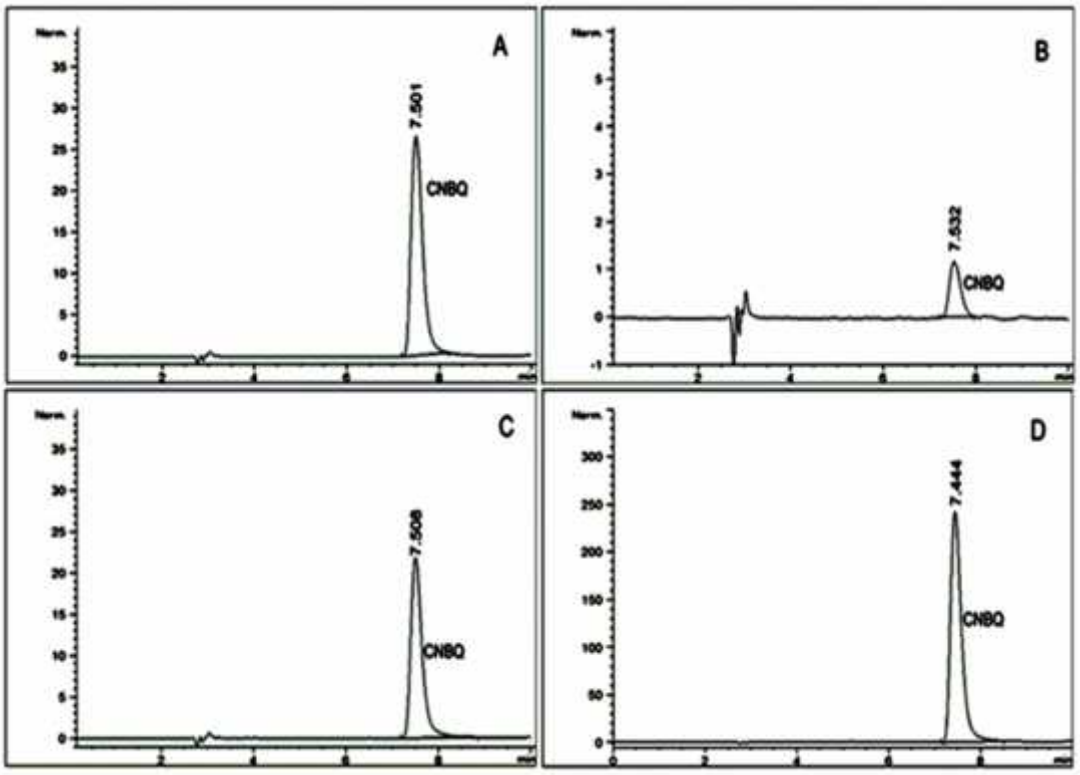


Figure10. HPLC Chromatograms obtained in solubility studies; A: Standard Chromatogram for FB dissolved in acetonitrile and water (50:50), B: Sample Chromatogram for FB dissolved in water, C: Standard Chromatogram for hydrochloride salt of CNBQ dissolved in acetonitrile and water (50:50) and D: Sample Chromatogram for hydrochloride salt of CNBQ dissolved in water.

5.3.6 Conclusion

The method adopted for determination of solubility of the CNBQ drug lead is specific, precise, linear, accurate, and rugged enough. Test sample solution is found to be stable up to 48 hours at room temperature. Hence, this method can be considered valid for its intended purpose to establish the quantity of CNBQ with consistent and reproducible results. The RP-HPLC method and the technique can be used for the determination of further solubility study of the drug lead CNBQ and also the same class of the drug.

5.4 PURITY DETERMINATION AND STABILITY STUDY

5.4.1 Abstract

The purpose of this study was to evaluate the purity and stability of a novel antimalarial drug lead, CNBQ (free base; FB) and its HCl salt using reverse phase-high performance liquid chromatography (RP-HPLC). Chromatographic separation of drug lead CNBQ, was successfully achieved on a C-18 column (4.6mm x 250mm, 5.0µm) in a gradient separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with Phosphoric Acid. The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325nm. Stability of both FB and its salt were carried out in different mediums and samples were analyzed using reverse phase-high performance liquid chromatography (RP-HPLC). The solutions of both form were stable in acid (0.1M HCl, pH 1.0 buffer), base (0.1M NaOH, pH 12.0 buffer), and water, but unstable in 0.3%H_2O_2. The hydrochloride salt form was found to be more pure and stable than the FB.

5.4.2 Introduction

Potency can be defined as the amount of drug present in a drug sample and is determined by RP-HPLC, ultraviolet-visible spectrophotometry and Potentiometric titration in general. Potency of the drug substances/product may vary with time under the influence of a various environmental factors such as temperature, humidity and drug excipients interaction, which lead to generation of impurities, which are may be toxic and causes undesirable effects on patients. Therefore, regulatory agencies recommend the use of stability-indicating methods for the analysis of stability samples for the determination of potency and its impurities generated during its shelf life. Stability indicating methods are defined by the US Food and Drug Administration as quantitative analytical methods that "are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product" and

"will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured."[19,20]

The stability of drug candidates is another indispensable physicochemical property to be considered during drug discovery process. The stability screening provides overall stability of compounds in various pharmaceutical conditions and also helps to identify impending hurdles during the development process.[8] ICH guidelines requires the parent drug to be tested under stress conditions such as the effect of pH, temperature, humidity, light, and oxidizing agents.[21] Stability of compounds in solution (at different pH) is required to formulate the solution dosage forms. Oxidation being the most common degradation pathways for organic compounds, analysis of oxidative stability is one of the crucial steps in the drug discovery and development. Photostability study is important for functions like handling, packaging, and labeling of compounds.[22]

5.4.3 Materials and Methods

5.4.3.1 Materials:

Methanol, sodium hydroxide, hydrochloric acid, hydrogen peroxide, triethylamine, sodium phosphate dibasic anhydrous and phosphoric acid were purchased from Fisher Scientific, whereas formic acid from Sigma-Aldrich. HPLC/LC-MS grade water was purchased from Fisher Scientific. The deionized water used was further purified by filtration and degassing. The drug lead mentioned in the figure 2 was synthesized in the laboratory.

5.4.3.2 Experimental Methods

Potency for both FB and salt were determined using area normalization method from the HPLC chromatograms. Stability indicating nature of the following RP-HPLC method was confirmed by peak purity checking of the HPLC chromatograms obtained by forced degradation studies. Both FB and salt were treated with different medium (0.3% H_2O_2, 0.1 M HCl, 0.1 NaOH, water, and buffer: pH 1.0-12.0) for 48 hours, also the samples were treated with sunlight and heat (60°C) for seven days.

Chromatographic conditions:

Chromatographic separation of CNBQ, and its degradants were estimated by C-18 column (4.6mm x 250mm, 5.0µm particle size), purchased from Waters Corporation. A gradient separation mode (Table 5) with mobile phase consisting of 0.1% of triethylamine in methanol as organic phase and 0.02 M dibasic sodium phosphate (anhydrous) at pH 3.5 adjusted with Phosphoric Acid as buffer. The instrumental set-up included the flow rate which was maintained at 1.0 ml/min, and temperature of the column oven at 40°C. The injection volume was set to 10 µl and the runtime for 25 minutes. Effluent was detected at 325nm (based on the λ of Isoquinoline). The peak purity of the sample chromatograms were obtained using photodiode array detector (PDA, G1315D-Agilent).

Table 5: Mobile phase gradient elution mode

Time (Min)	**%Buffer (0.02 M Na_2PO_4 at pH 3.5)**	**%Organic Phase (0.1% TEA in Methanol)**
0	48	52
6	48	52
15	10	90
20	48	52
25	48	52

5.4.4 Analytical Method Validation Report

To validated the test method, different analytical performance parameters such as system suitability, linearity, specificity, limit of detection, limit of quantitation, forced degradation study, accuracy, precision, solution stability and robustness were determined according to ICH issued analytical method validation guidelines Q2 (R1).[16]

5.4.4.1 System Suitability

To assess system suitability of the proposed method, repeatability, theoretical plates, tailing factor, and retention time of six replicate injections of standards were measured. Percentage relative standard deviation (%RSD), tailing factor and theoretical plate values were calculated in each case. The results (Mean ± %RSD of six replicates) of the chromatographic parameters in table 6 indicating the good performance of the system.

Table 6: Chromatographic characteristics of system suitability

Parameter	**Value (Mean ± %RSD)**	
CNBQ	**FB**	**Hydrochloride Salt**
%RSD of Peak area	113.265± 0.82%	91.086± 1.03%
Tailing factor	1.3 ± 0.1	
Theoretical plate	3457 ± 1.23	

5.4.4.2 Specificity

The specificity of the developed method was determined by blank analysis and checking peak purity of the test solution. From this study, it is observed that each component gave response separately with respect to retention time and passed peak purity. No interference due to blank was observed in the chromatogram for blank solution (Figure 11). Therefore, the method is considered to be specific.

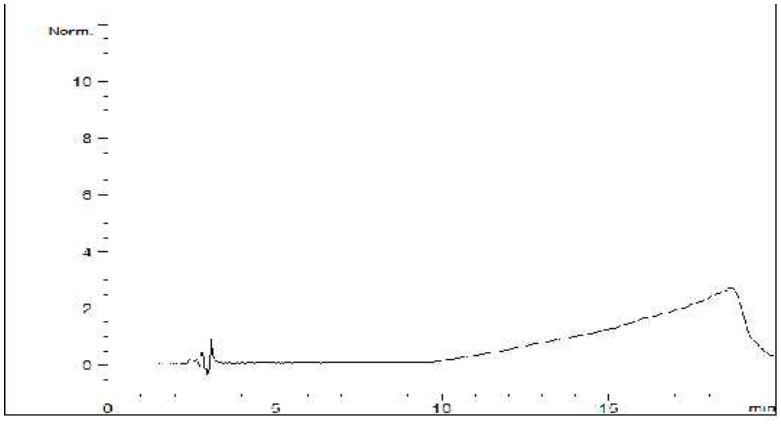


Figure 11. HPLC Chromatograms; Blank: acetonitrile and water (50:50)

5.4.4.3 Linearity

The linearity was evaluated by analyzing different concentration of standard solutions of FB and its salt over the concentration range of 0.2 µg/ml (limit of detection) to 9.6 µg/ml by linear regression analysis, which was then evaluated by the least-square regression analysis. In both case the correlation coefficient were within the limit of not less than 0.99 (FB: $y = 11.158x - 0.7561$, $R^2 = 0.9998$ and Salt: $y = 14.567x - 1.6543$, $R^2 = 0.9987$). Thus the method is considered to be linear.

5.4.4.4 Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated in accordance with the 3.3s/m and 10s/m criteria, respectively, according to ICH Q2 (R1) recommendations, where 's' is the standard deviation of the peak area and 'm' is the slope of the calibration curve, determined from linearity investigation. Figure 12 represents the HPLC Chromatograms obtained from calculated limit of detection level (0.2 µg/mL), and B: limit of quantitation level (0.6 µg/mL).

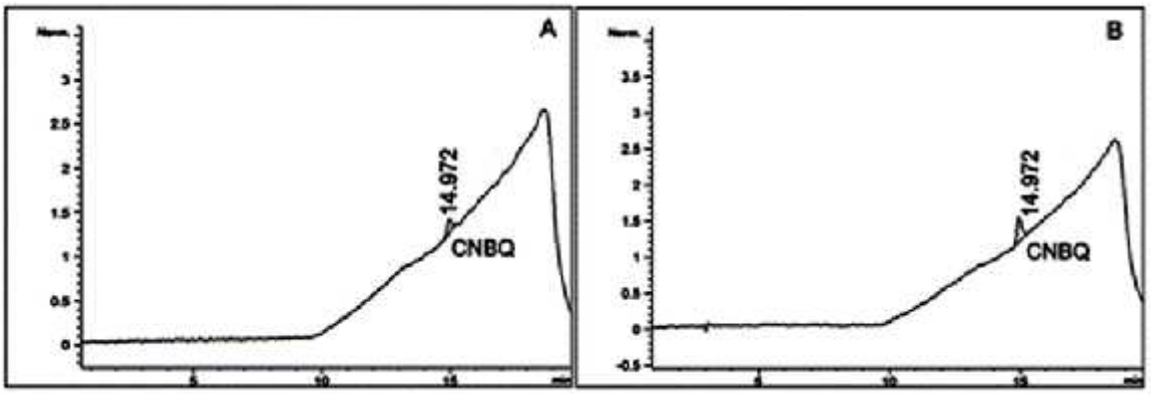


Figure 12. HPLC Chromatograms for; A: limit of detection level (0.2 µg/mL), and B: limit of quantitation level (0.6 µg/mL).

5.4.4.5 Forced Degradation Study

Forced Degradation study for both FB and salt were carried out using different medium (0.3% H_2O_2, 0.1 M HCl, 0.1 NaOH, water, and buffer: pH 1.0-12.0) for 48 hours, also the samples were treated with sunlight and heat (60°C) for seven days.

Stock solution (0.08 mg/ml) of FB and salt were prepared to check the solution stability in the above medium.

Table 7: Sample preparation details for stability study.

Medium	Sample ID	Substrate Added (ml)	Medium Added (ml)	Final Volume (ml)	Final Concentration (mg/ml)
0.3 % H_2O_2	Blank	N/A	0.1 ml	10 ml	N/A
	Test Sample	1 ml	0.1 ml	10 ml	0.008
0.1 M HCl	Blank	N/A	1 ml	10 ml	N/A
	Test Sample	1 ml	1 ml	10 ml	0.008
0.1 M NaOH	Blank	N/A	1 ml	10 ml	N/A
	Test Sample	1 ml	1 ml	10 ml	0.008
H_2O	Blank	N/A	1 ml	10 ml	N/A
	Test Sample	1 ml	1 ml	10 ml	0.008
pH (1.0-12.0)	Blank	N/A	1 ml	10 ml	N/A
	Test Sample	1 ml	1 ml	10 ml	0.008

After adding substrate to the medium, the samples were kept in water bath (80°C) for 30 minutes and were left at room temperature for next 48 hours. The samples were then dissolved in acetonitrile and water (50:50) to obtain a concentration of 0.008 mg/ml that were analyzed using RP-HPLC. For solid-state stability, FB and salt were kept in the sunlight and in oven (60°C) for seven days. These samples (0.008mg/ml) were then analyzed using similar chromatographic conditions. RP-HPLC chromatograms and results obtained during forced degradation study are presented in the results and discussion section.

5.4.4.6 Accuracy

Recovery test of the proposed method was studied at 80%, 100% and 120% of nominal test concentration and the percent recoveries were calculated. All the results were found within the acceptable limit of 98.0% to 102.0%.

Table 8: Accuracy studies of FB and salt of CNBQ.

Sample Name	%recovery	
	FB	**Salt**
80% Sample 1	101.4	99.6
80% Sample 2	101.1	99.3
80% Sample 3	99.8	99.3
100% Sample 1	99.7	100.5
100% Sample 2	100.3	98.9
100% Sample 3	100.8	98.5
120% Sample 1	98.9	101.3
120% Sample 2	98.5	101.4
120% Sample 3	101.2	99.8
Mean	100.2	99.8
Standard Deviation	1.04	1.02
%RSD	1.04	1.02

5.4.4.7 Precision

The repeatability (intra-day precision) of the method was assessed by performing replicate measurements (n=6) of both FB and salt of CNBQ and the intermediate precision (inter-day precision) of the method was assessed by performing replicate measurements (n=6) of both FB and salt of CNBQ form the same batch by different analyst on different dates. The results were expressed as %RSD of the measurements. %RSD of the repeatability were found 1.14% and 1.66% and %RSD of intermediate precision were found 0.79% and 0.57%, respectively. All the results were found within the acceptable limit of not more that 2.0%. Thus the method is considered to be precise.

5.4.4.8 Sample Solution Stability

To check the solution stability, test sample solution was kept in room temperature and light for two days and measured the peak area of the analyte after specific intervals of

3, 6, 12, 18 24, 36 and 48 hours and the percent difference between initial results were calculated. The test sample solution of both FB and salt of CNBQ were found to be stable up to 48 hours at room temperature because the percent differences between initial results were 0.39% and 0.63% at 48 hours, respectively.

5.4.4.9 Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. To determine the robustness of the current method, the pH of the buffer solution was assessed at 3.4 and 3.6 instead of 3.5. The effect of flow rate was studied at 0.9 and 1.1 mLmin-1 instead of 1.0 mLmin-1. The effect of column temperature was studied at 35° and 45°C instead of 40°C. The system suitability parameters under these conditions was calculated in all cases and found well within the acceptable limit (%RSD of peak area NMT 2.0%, Tailing factor NMT 2.0, and Theoretical plate NMT 2000).

5.4.5 Results and Discussion

Samples were analyzed by stability indicating RP-HPLC method equipped with a peak purity analyzer (photodiode array). The stressed samples were compared to the unstressed sample (control). The method is found to be highly sensitive (0.2 µg/ml, limit of detection) and linear ($R^2 = 0.99$) over a range of limit of detection to 120% of the target concentration level (Figure 12). Degradation peaks were well resolved from the main peak (CNBQ). The peak purity of the principal peak was greater than 0.999 in all stress conditions suggesting that there was no interference of degradants with the principal peak.

Potency for both FB and salt were determined using area normalization method from the HPLC chromatograms. The hydrochloride salt form was found to be more pure and stable than the FB. The results (Mean ± %RSD of three replicates) of the purity are presented in Table 9.

Table 9: Purity results of FB and salt of CNBQ.

%Purity of CNBQ	FB	HCl Salt
Sample 1	97.2	100.0
Sample 2	96.9	100.0
Sample 3	97.5	100.0
Mean	97.2	100.0
%RSD	0.31	---

Figure 13-27 represents the HPLC Chromatograms obtained in forced degradation studies of FB of CNBQ and its hydrochloride salt. From the blank chromatograms (Figure 13) it is clearly seen that diluent and mobile phase has no interference in the studies because there is no co-eluting peak at the retention time of CNBQ (~14.75 min) in the blank chromatograms. FB has one process related impurity (Cyclen Trisquinoline) at the retention time of about 16.75 minute, which completely disappears in the salt form. Both FB and salt are found to be stable in all stress conditions except oxidation, as there were no degradants observed in the HPLC chromatograms, except Figure 17 and 24. Ten and seven degradants were observed during oxidative degradation of FB and its salt, respectively. Therefore, both FB and salt are susceptible to oxidative degradation, and the free base is more susceptible to oxidative degradation compared to the salt form.

Stability testing provides prophecy about the quality of a drug substance with time due to variation in environmental factors such as temperature, humidity, and light. It also helps to establish the recommended storage conditions and shelf-life of the drug substance.[21,22] The preliminary studies have shown that CNBQ can be stored at room temperature. Forced degradation studies have been proven to be less time consuming as compared to stability studies in predicting the possible degradants.[23] To confirm the storage conditions and shelf-life, long term, intermediate, and accelerated stability studies are needed.[24] Conducting degradation studies earlier in the drug development process will allow adequate time to collect information about the stability of the molecule. The stability indicating method developed during forced degradation studies

can be used for the analysis of samples generated from accelerated and long term stability studies. These studies are also helpful in the implementation of quality risk management by QbD approach[25] for formulation development and manufacturing process design, and to determine the storage conditions of the finish product.

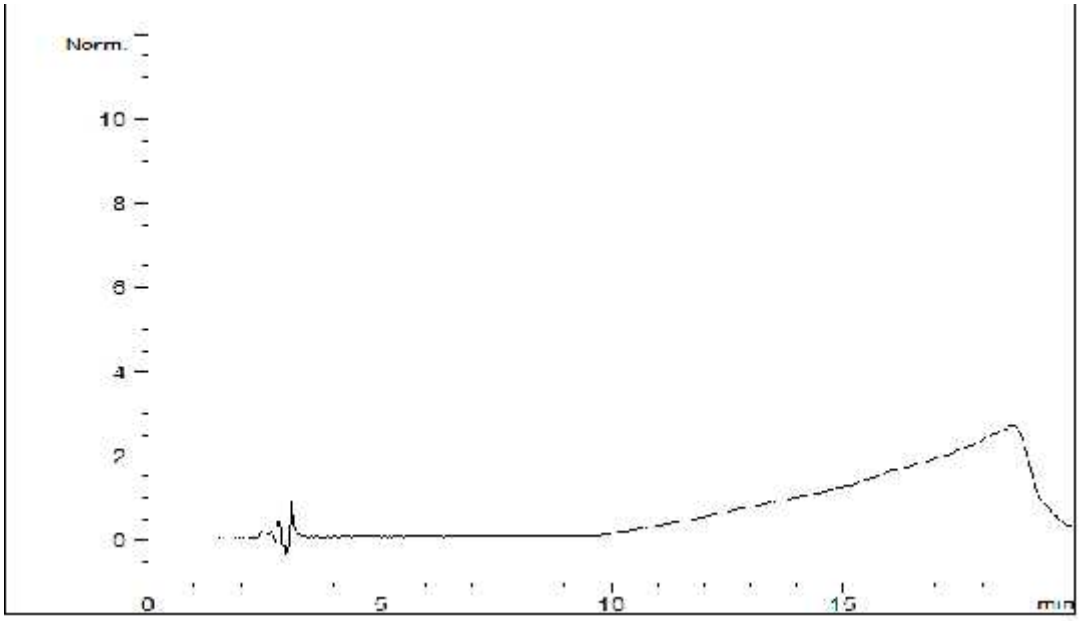


Figure 13. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Blank.

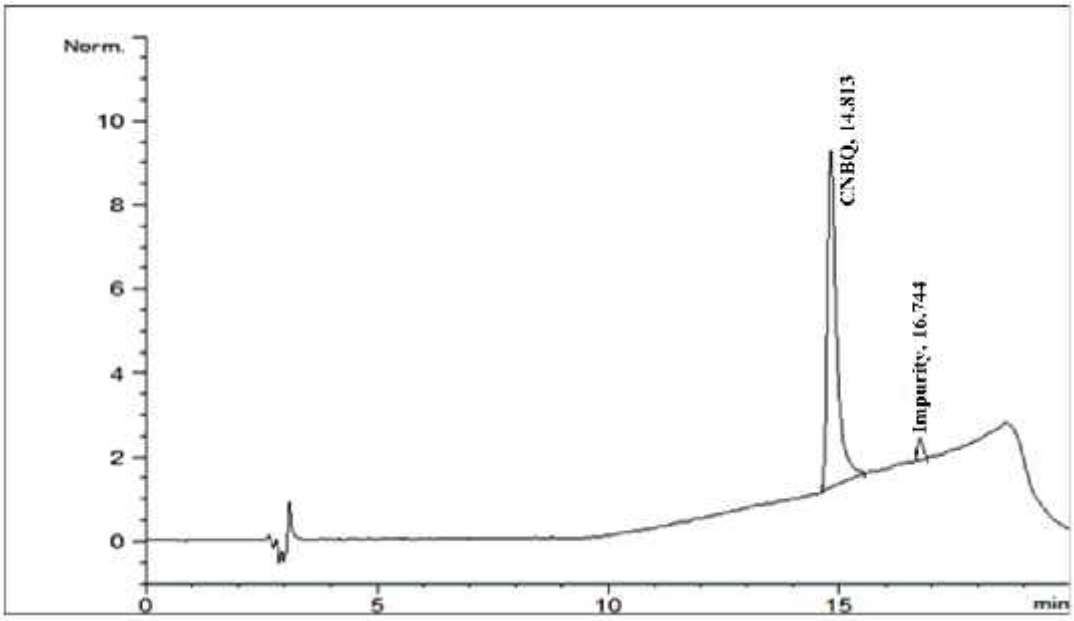


Figure 14. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; standard.

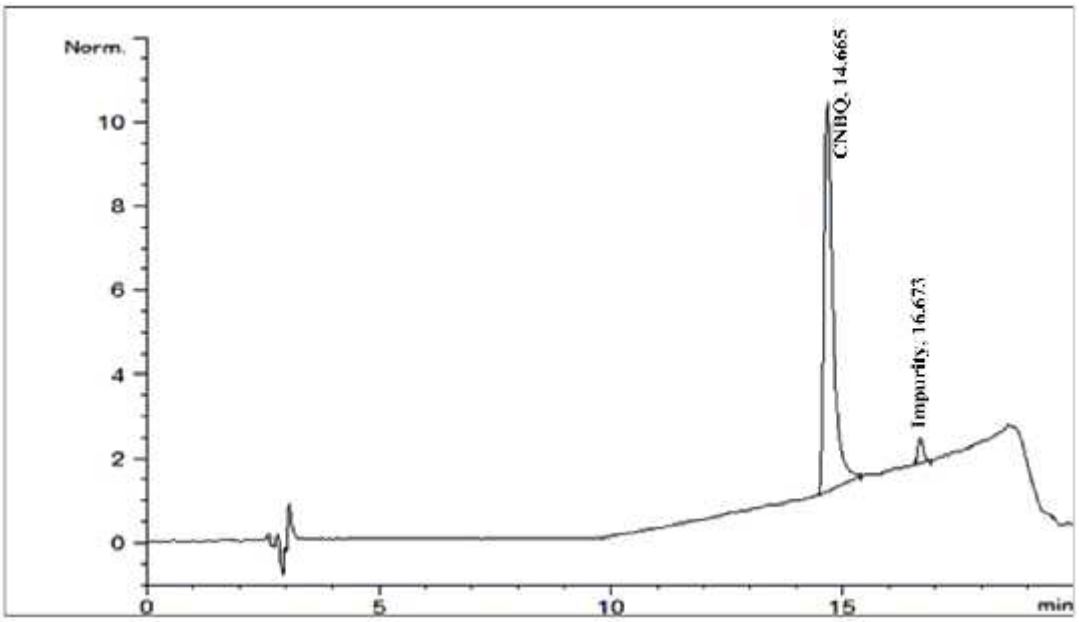


Figure 15. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Acid hydrolysis.

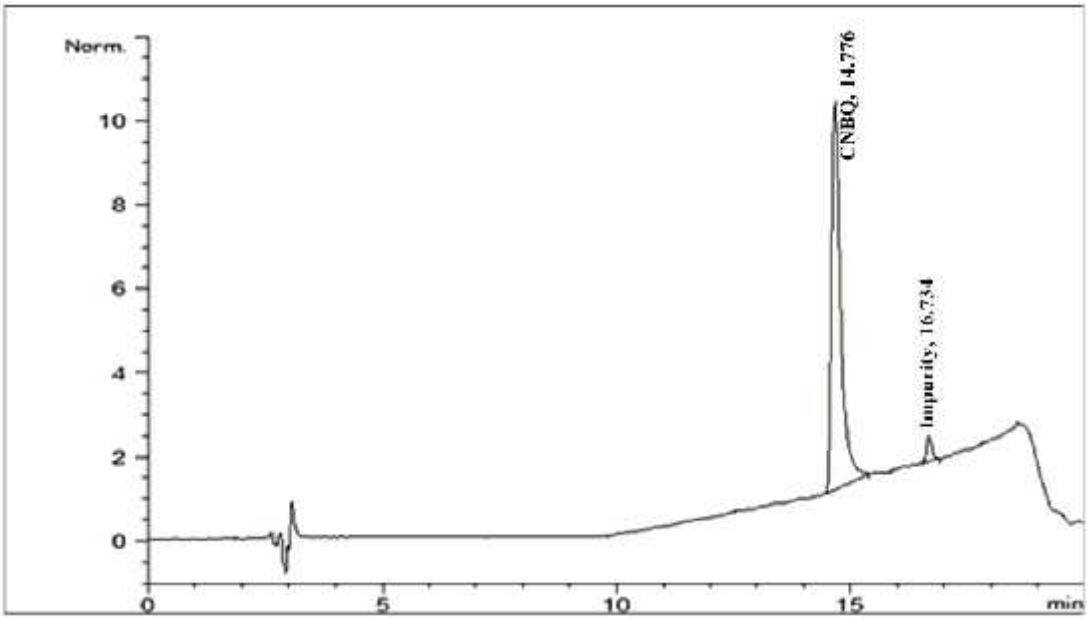


Figure 16. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Base hydrolysis.

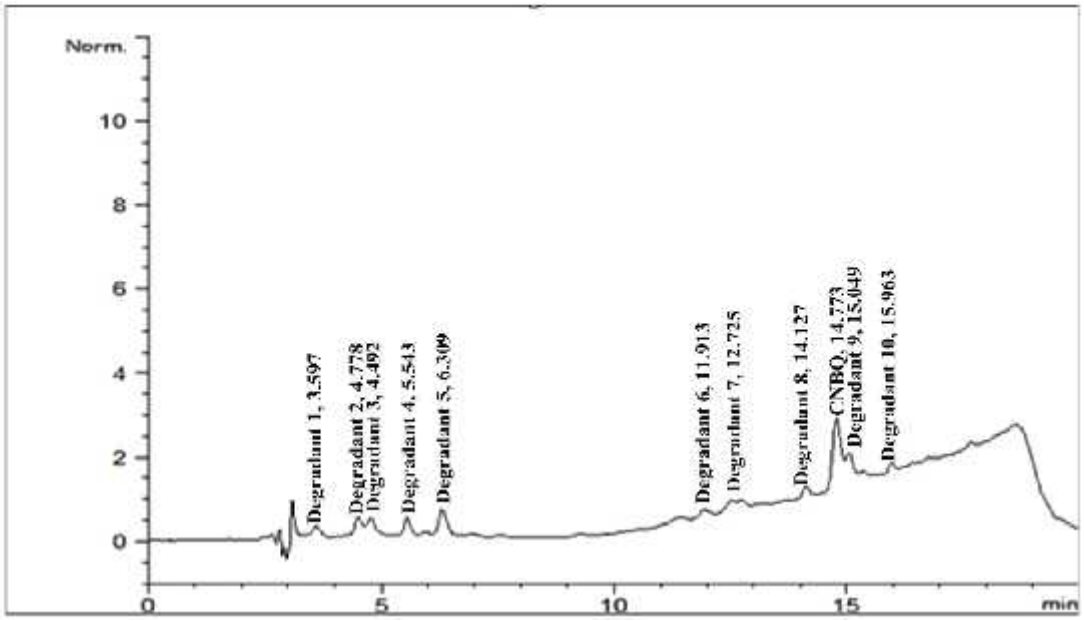


Figure 17. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Oxidation.

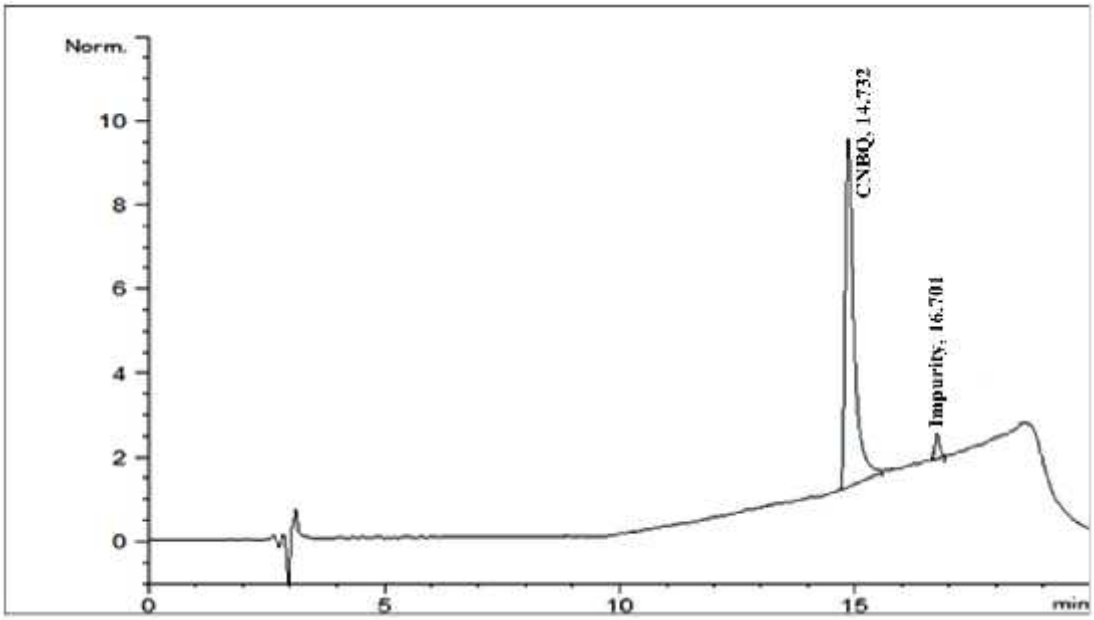


Figure 18. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Heat.

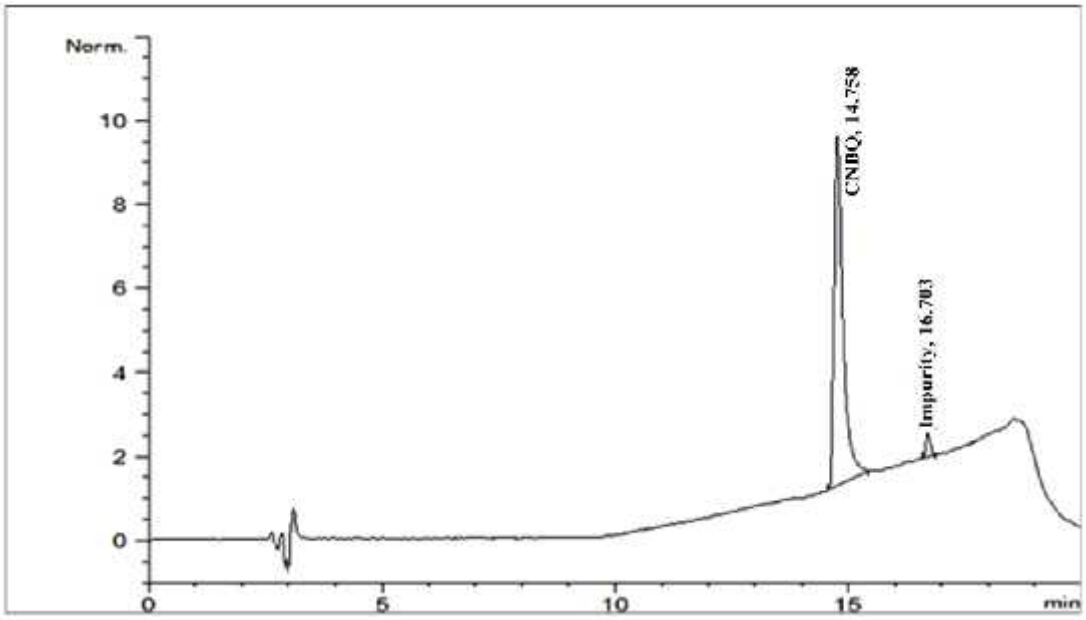


Figure 19. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Light.

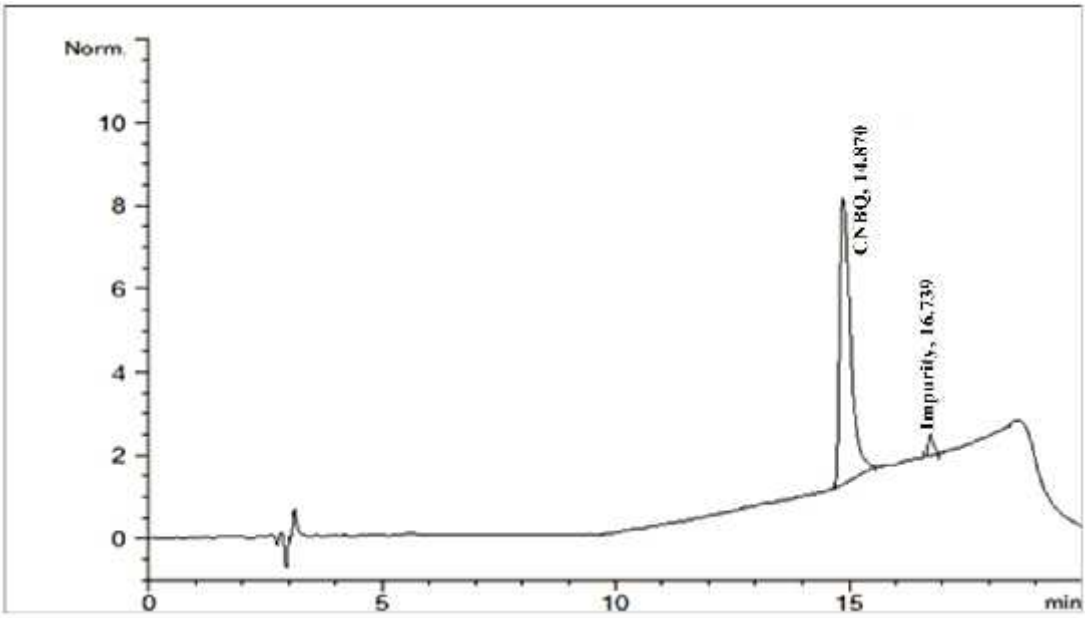


Figure 20. HPLC Chromatograms obtained in forced degradation studies of CNBQ FB; Buffer solution at pH 12.0.

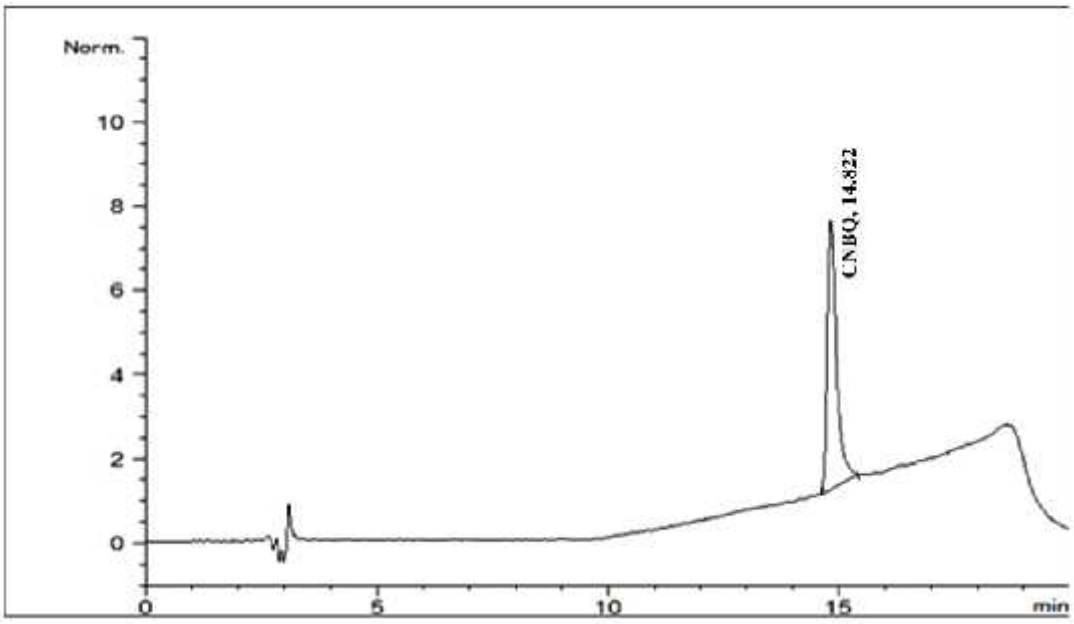


Figure 21. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; standard.

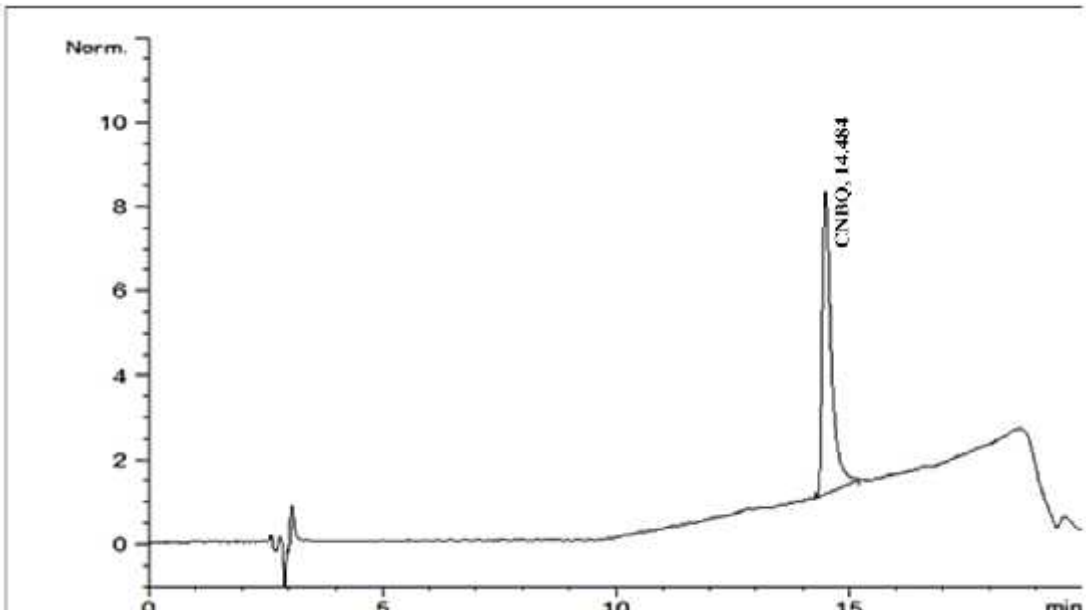


Figure 22. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Acid hydrolysis.

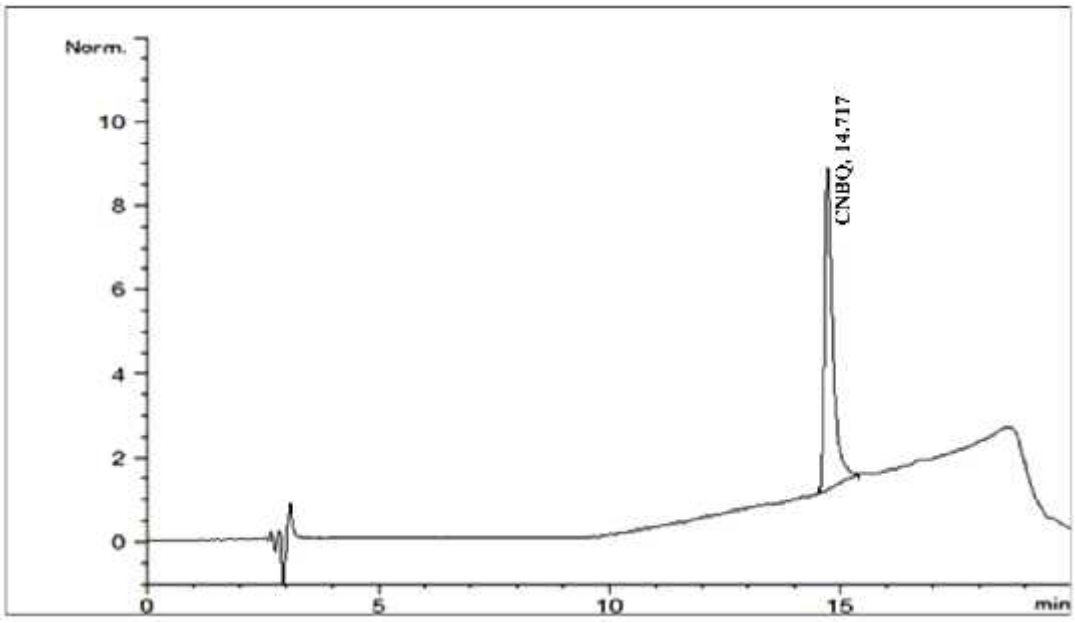


Figure 23. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Base hydrolysis.

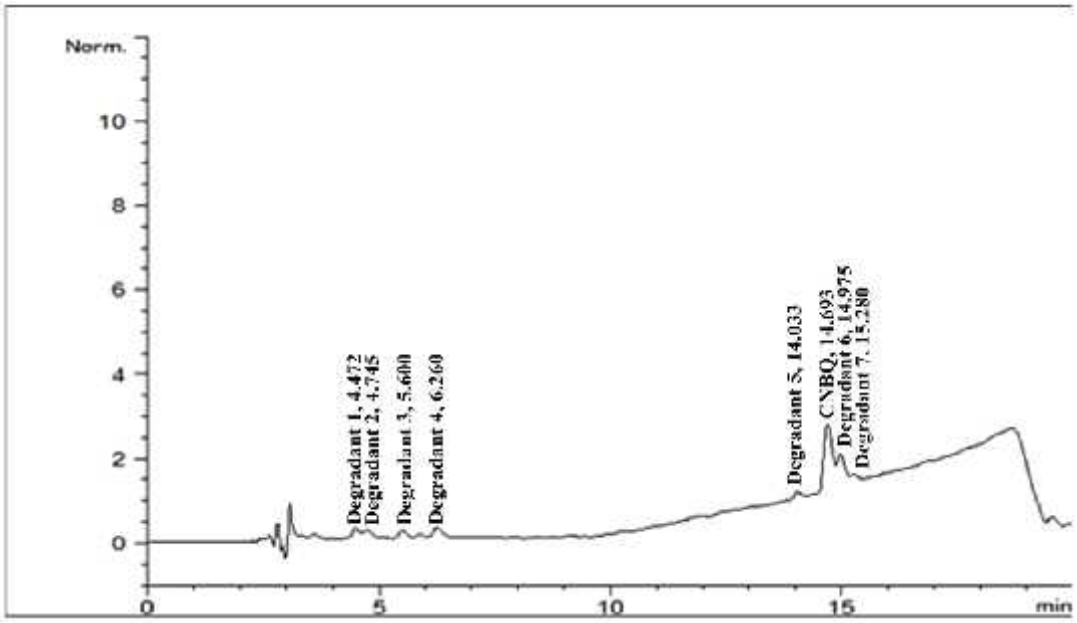


Figure 24. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Oxidation.

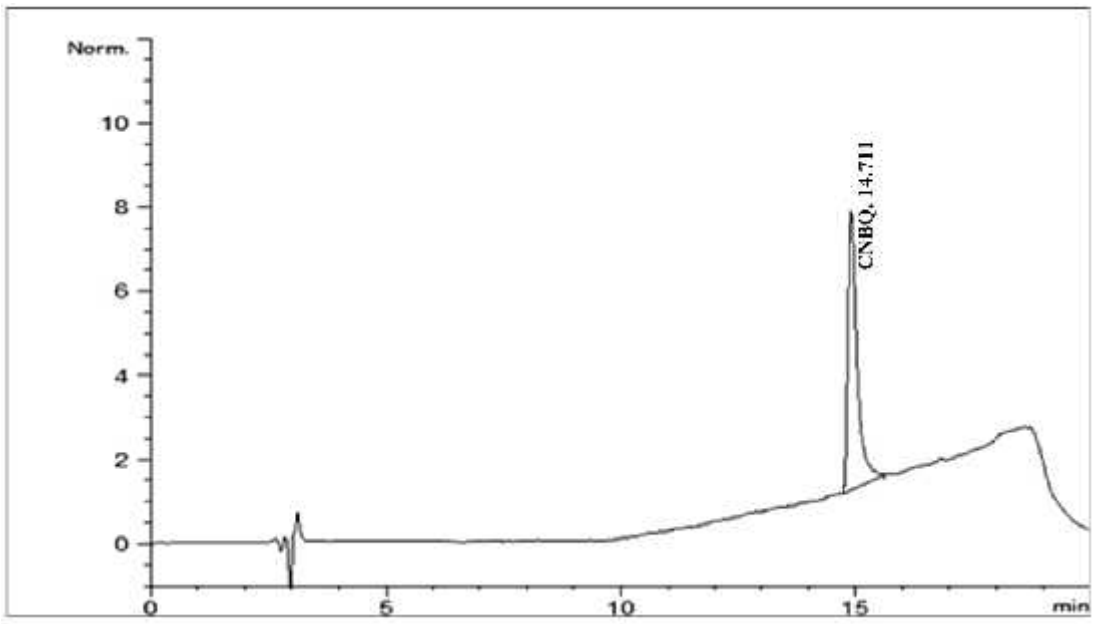


Figure 25. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Heat.

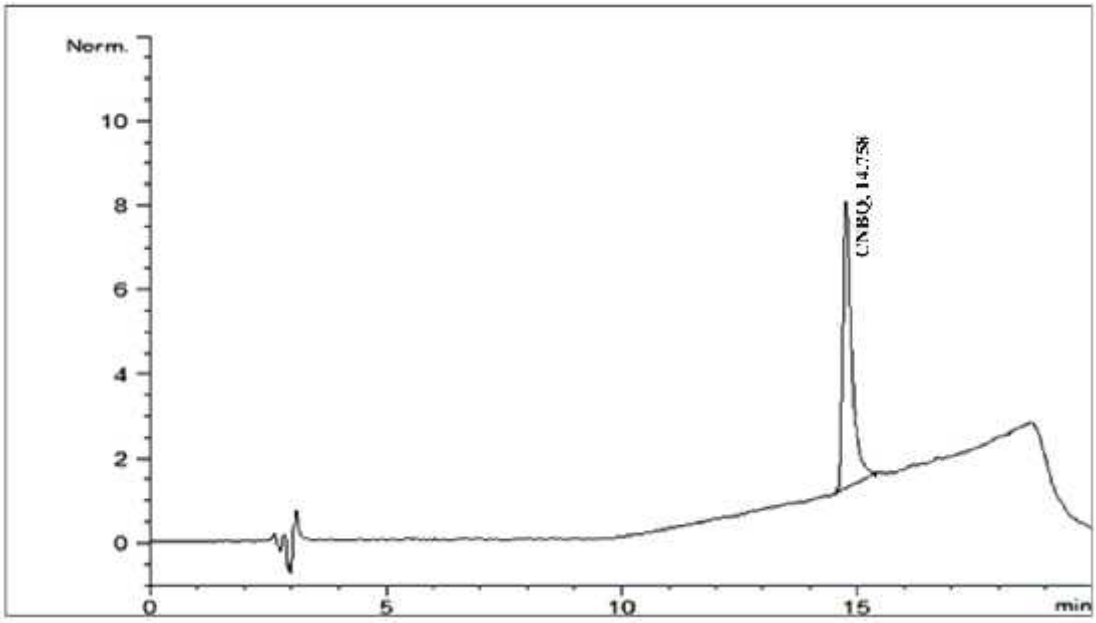


Figure 26. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Light.

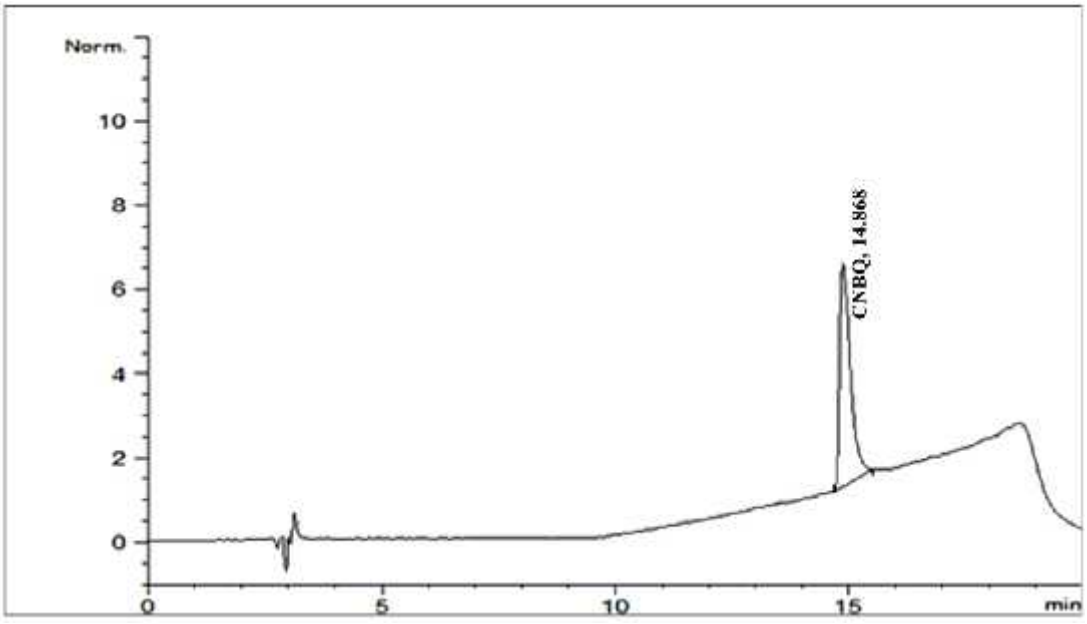


Figure 27. HPLC Chromatograms obtained in forced degradation studies of hydrochloride salt of CNBQ; Buffer solution at pH 12.0.

5.4.6 Conclusion

Stability of drug products is the fundamental parameter that should be evaluated before any drug development from its discovery stage. The stability study assists in evaluating appropriate dosage form that would be required to resolve any complications during the process. The method adopted for determination of purity and stability of the CNBQ drug lead is specific, precise, linear, accurate, and rugged enough. Test sample solution is found to be stable up to 48 hours at room temperature. Hence, this method can be considered valid for its intended purpose to establish the quantity of CNBQ with consistent and reproducible results. The RP-HPLC method and the technique can be used for the determination of further purity and stability of the drug lead CNBQ and similar class of the drugs.

5.5 METABOLIC (IN VITRO) STABILITY STUDY

5.5.1 Abstract

The metabolic stability of CNBQ was determined using human liver microsome (HLM) and specific cytochrome P450 enzyme (CYP2C8) taking the clinically used antimalarial drug chloroquine as a positive control. Chromatographic separation of drug lead CNBQ, CQ and their metabolites were successfully achieved on a C-18 column (4.6mm x 250mm, 5.0µm) in an isocratic separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with phosphoric acid in the ratio of (60:40, v/v). The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325nm. Peak purity data was obtained using photodiode array (PDA) detector. All the assays were conducted in 0.5M phosphate buffer at pH 7.4. In general the metabolic reaction was initiated by adding 1mM NADPH and 0.5 mg of enzyme. Incubations were done with time frequency of 0hr, 1hr, and 2hrs at 37^{o}C and the reactions were terminated by adding acetonitrile in the equal amounts of the assay mixture taken. The samples were centrifuged for 15min at 10,000 x g at 4^{o}C and an aliquot of the supernatant was subjected to analysis using HPLC as well as LC-MS to confirm the masses of the drug and/or metabolite (s), if any. While chloroquine was found to be metabolized in a predictable manner by both HLM and CYP2C8, the drug lead was metabolically stable at similar experimental conditions. This study demonstrated that the new drug lead is worth conducting further preclinical evaluations.

5.5.2 Introduction

With the arrival of new combinational drug synthesis, the need for an expeditious evaluation of drug safety has become a paramount topic in drug discovery. The duration and impact of drug discovery revolving around drug absorption, distribution, metabolism and excretion will be evaluated according to their determined metabolic stability. The major organ for drug metabolism is liver that contains major drug

metabolizing enzyme called cytochrome P450 (CYP) system.[26] Drug metabolism refers to the susceptibility of compounds to biotransformation that depends on the presence of groups in the molecule those are open to enzyme catalyzed transformation.[27] Drug metabolism can be divided into two phases, Phase I and phase II. Phase I involves oxidation, reduction and hydrolysis reactions, which are catalyzed by the CYP and flavin containing monooxygenases (FMOs) whereas Phase II involves conjugation reactions catalyzed by metabolic enzymes like UDP-glucuronyltransferases (UGTs) and sulfotransferases.[28,29]

Recognizing the metabolites of drugs is of paramount importance in drug discovery and development. The identification of drug metabolites in the early stages of the drug discovery is important in the development processes. The analytical tools like liquid chromatography-mass spectrometry (LC-MS) and HPLC play prominent role in these processes. Through this process of identification, the pharmacokinetic profiles can be assessed that are highly significant in detecting safety and efficacy of the drug leads before they are progressed to the clinical trials.

The investigation for metabolites take an advantage of the fact that majority of drug metabolites can be classified as predictable as they are formed from common accepted biotransformation reactions. However, there are many other illustrations of primary metabolites that are formed from uncommon reactions and are, therefore, not easily predictable. Molecular masses of predicted metabolites (*m*/*z* values) can be estimated based on mass shifts from the parent drug. For example, Chloroquine is the major antimalarial drug that used in the treatment and prophylaxis of malaria, the protonated molecular mass of the metabolite of chloroquine, desethylchloroquine (DCQ), is 292m/z to that of the parent drug 320m/z. Evaluation of some expected metabolites can be achieved by the acquisition of the complete MS spectrum using various MS instruments and also by extracted ion chromatography (EIC).[29,30]

The main purpose of the present study was to determine the metabolic stability of a of newly discovered cyclen bisquinoline antimalarial drug lead CNBQ by RP-HPLC and LC-MS techniques. CNBQ was shown to be highly effective antimalarial agent both in

vitro and in vivo and was found to work by inhibiting β-hematin formation.[31] The present study was thus aimed at determining the *in vitro* metabolic stability and identifying potential metabolites by HPLC and LC-MS techniques using HLM and CYP enzymes. The study was designed based on the known metabolic pathway of the related clinically used drug chloroquine (CQ)[32-35] and thus utilized both pooled human liver microsomes as well as specific isozyme CYP2C8.

5.5.3 Materials and Methods

5.5.3.1 Materials:

Chloroquine diphosphate purchased from Pfaltz & Bauer. Anhydrous NADPH with assay 93-100% (HPLC), was purchased from Sigma Aldrich. 0.5M Potassium phosphate buffer pH 7.4 was purchased from BD Gentest. Human liver microsome (HLM) was purchased from BD Biosciences. Human cDNA expressing specific cytochrome P450 enzyme (CYP2C8) was purchased from Corning. In the solvent system methanol Triethylamine, DMSO, and acetonitrile were purchased from Fisher Scientific. Sodium phosphate dibasic anhydrous and phosphoric acid were purchased from Fisher Scientific. Deionized water used was further purified by filtration and degassing. The drug lead mentioned in the Figure 2 were synthesized.

5.5.3.2 General Procedure:

The method used to determine the metabolism was a modified method obtained from BD Bioscience, which is summarized here. Metabolic stability study of a drug can be determined by treating it with the liver enzyme that correlates with the *in vivo* conditions. The assay mixture is prepared by the combination of substrate (0.01mM), enzyme (0.5mg), buffer (0.5M), and 1mM NADPH cofactor. The order of addition of the assay component also plays a major role in the stability study. Metabolism can be initiated by pre-warming or incubating the substrate, buffer, and the cofactor to 37^{o}C and then adding liver microsomes or CYP2C8 to the mixture. It is customary that the assay mixture should be thoroughly mixed. After the preparation of the total assay mixture (TAM), required volume of the sample is collected at different time intervals.

The reaction is then terminated using acetonitrile in equal volumes as that of the sample solution collected. The samples are then transferred into the microcentrifuge tubes, vortexed for 2 minutes and then centrifuged at 10,000 x g for 15 minutes. The purpose of centrifugation is to remove the protein. The sample solution is then separated from the protein pellet by taken the supernatants. These supernatant solutions are transferred into the HPLC autosampler vials and are analyzed according to the analytical method.

5.5.3.3 Metabolic Stability in HLM and CYP2C8

The HLM used was pooled and prepared from freshly frozen human tissues which were tested negative for pathogens using PCR. The HLM used in this study was comprised of 330 pmoles/mg of total P450 and 420 pmoles/mg cyt. b_5. It is a mixture of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 4A11, FMO, UGT1A1, UGT1A4, and UGT1A9 where the enzyme activity of CYP2C8, 3A4, and 2D6 was 82, 3200, and 110 pmoles/(mg x min) respectively. The volume of the enzyme per vial was 0.5ml and the protein content was 20mg/ml in 250mM sucrose. The CYP enzyme is comprised of a human CYP2C8 and human CYP-reductase co-expressed in *Escherichia coli*. The concentration of the CYP P450 is 1.0 nmol/ml and its protein concentration is 10.0 mg/ml.

To evaluate the metabolic stability of drug lead CNBQ, CQ was used as a positive control. For that we prepared sample, standard and blank for our drug lead CNBQ and CQ in duplicates as mentioned in the following table 10. All the ingredients in the Total Analytical Mixture (TAM) were taken in the order of their serial number.

Table 10: Total assay mixture using HLM and CYP2C8:

SL. No.	Ingredients	Sample		Standard	Blank
		Volume, 2 mL of TAM	Final Concentration	Volume, 2 mL of TAM	Volume, 2 mL of TAM
1	Substrate (1mM)	20 µl	0.01mM	20 µl	---
2	Buffer (0.5M)	400µl	100mM	400µl	400µl
3	NADPH	200 µl	1mM	---	200 µl
4	DI Water	1355 µl	---	1580 µl	1375 µl
5	Enzyme	25 µl	0.5mg	---	25 µl

Sample stock solutions (1mM) of Chloroquine diphosphate and Cylen Bisquinoline Hydrochloride Salt were prepared by dissolving in DI water. 0.5M potassium phosphate pH 7.4 (BD Biosciences Cat No. 451201) was used as buffer. Sample solutions were prepared by adding 20µl of substrate, 400µl of the buffer solution, 200µl of the NADPH solution and 1355µl of water. Standard solutions were prepared by adding 20µl of substrate, 400µl of the buffer solution and 1580µl of water. Blank solutions were prepared by adding 400µl of the buffer solution, 200µl of the NADPH solution and 1375µl of water.

All the above solutions were then pre-incubated for 5 minutes in a shaking water bath at 37°C. Reaction was initiated by adding 25µl of the enzyme (from Corning HLM-452161 and CYP2C8-456252). These TAMs were mixed thoroughly by inverting a couple of times and returned to the water bath. Immediately, at time t= 0hr, 500µl of the TAM was taken into a centrifuge tube and the reaction was terminated by placing it on ice bath and adding 500µl of acetonitrile. The remaining TAMs were warmed in a shaking water bath at 37^{o}C for 2hrs and the same procedure was followed for the samples at time t= 1hr and t=2hr. The incubation mixtures were then centrifuged for 15 minutes at 10,000 x g at 4^{o}C. About 500 µl aliquots of the supernatant were collected and subjected to analysis using the RP-HPLC method. After running HPLC the samples were frozen and then lyophilized to remove the solvent. The dried samples were then dissolved in methanol and water (50:50 v/v) for LC-MS analyses.

The retention times were noted and the amounts of drugs metabolized were calculated according to their percentage peak area using the equation: %peak area of the metabolite / (%peak area of the drug + %peak area of the metabolite). The mass of the drug and the metabolite were determined by using LC-MS.

5.5.3.4 RP-HPLC and MS Method

Chromatographic separation of drug lead CNBQ, CQ and their metabolites were successfully achieved on a Waters X-Bridge C-18 column (4.6mm x 250mm, 5.0µm particle size, part no. 186003117) purchased from Waters Corporation in an isocratic separation mode with mobile phase consisting of 0.1% of triethylamine in methanol and 0.02 M dibasic sodium phosphate at pH 3.5 adjusted with Phosphoric Acid in the ratio of (60:40, v/v). The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325nm (based on the λ, Figure 28).

LC-MS system used was Shimadzu prominence LC 20AT, equipped with Degasser (DGU- 20A5), Auto sampler (SIL-20AHT), mass spectrometer (LC-MS 2020) in an isocratic separation mode with mobile phase consisting of methanol and LC-MS Grade water in the ratio of (50:50, v/v). The flow rate was maintained at 1.0 ml/min. Data Acquisition was performed by Lab Solutions Real Time Analysis Software, implemented in the chromatographic system.

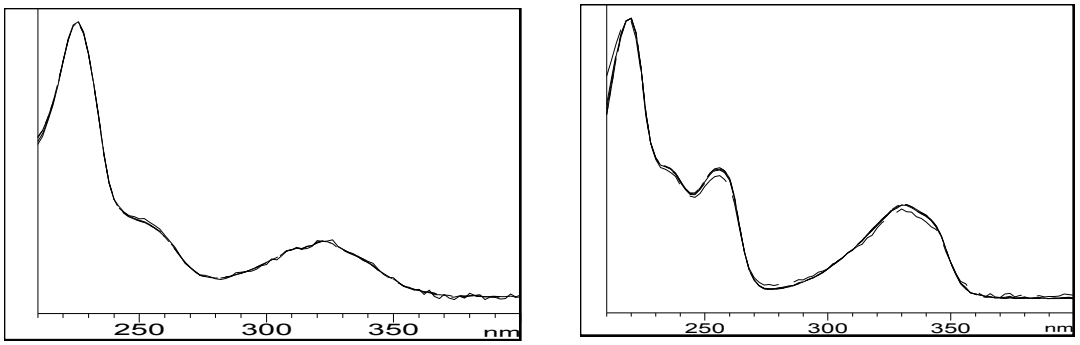


Figure 28: Spectrum of A: Chloroquine Diphosphate (CQ) and B: Cyclen Bisquinoline (CNBQ).

5.5.4 Analytical Method Validation Report

Specificity of the method was carried out by checking the blank and control sample in RP-HPLC, and confirmed by LC-MS analysis. The peak purity data were obtained using photodiode array (PDA) detector in the sample chromatograms to check the co-elution of any metabolites with principal peak. From the study, it is observed that each component gave response separately with respect to retention time and passed peak purity. The reproducibility and accuracy of the method was confirmed by the preparation of the duplicate sample along with the replicate injection. The solution stability of the method was confirmed by injecting the standard sample along with the sample solution at the same time interval. The method was found linear over the concentration range of 0.1–8.0 µg/ml ($y = 41.643x + 0.6407$, $R^2 = 0.9997$) for drug lead CNBQ. Sensitivity of the method was calculated based on the standard deviation of the response and the slope as per ICH guideline. Limit of detection value for drug lead CNBQ was obtained 0.18 µg/ml. Percentage of the metabolites were calculated using area normalization procedure.

5.5.5 Results and Discussion

5.5.5.1 Results using HLM

Under provided experimental conditions it was observed that, CQ was metabolized by HLM into the metabolite DCQ and contrarily our experimental drug lead was metabolically stable. The metabolite was identified by HPLC (Figure. 35 & 36) and also by LC-MS (Figure. 30) reconfirming the results. Both drug (320 m/z) and metabolite (292 m/z) masses were observed in the LC-MS analysis of the samples. According to the area under curve ratios, CQ was converted to about 4.29% of its N-deethylated metabolite after 1 hour of incubation and 6.61% after 2 hours of incubation. On the other hand, the drug leads (495m/z) was found to be metabolically stable in both HPLC and LC-MS analyses. The retention times of the drug lead and the CQ at time t=1hr and t=2 hr are shown in the Table 11. All the samples were estimated in duplicates and the results shown are the average of the two trials.

Table 11: Data obtained from HPLC and MS for the drug using HLM

	Time t= 1 hr			Time t= 2 hr		
Drugs	**RT**	**Metabolism**	**MS-(m/z)**	**RT**	**Metabolism**	**MS-(m/z)**
CNBQ	13.135	NO	495	13.10	NO	495
CQ/DCQ	5.460/4.480	YES	320, 292*	5.470/4.508	YES	320, 292*

*The percent of CQ metabolized using HLM at time t=1hr and 2hrs was 4.29% and 6.61%, respectively.

5.5.5.2 Results using CYP2C8

Under provided experimental conditions it was observed that CQ was metabolized by CYP2C8 into the metabolite DCQ and contrarily our experimental drug lead B was metabolically stable. Using CYP2C8 about 2.23% of CQ was transformed into DCQ after 1 hr of incubation and 2.89% after 2 hrs of incubation. The drug lead (495m/z) was metabolically stable at similar experimental conditions (Figure. 32). The retention times of the drug lead and the CQ at time t=1hr and t=2 hr are shown in the Table 12. All the samples were estimated in duplicates and the results shown are the average of the two trials.

Table 12: Data obtained from HPLC and MS for the drugs using CYP2C8

	Time t= 1 hr			Time t= 2 hr		
Drugs	**RT**	**Metabolism**	**MS-(m/z)**	**RT**	**Metabolism**	**MS-(m/z)**
CNBQ	13.145	NO	495	13.129	NO	495
CQ/DCQ	5.486/4.533	YES	320, 292*	5.432/4.517	YES	320, 292*

*The percent of CQ metabolized using CYP2C8 at time t=1hr and 2hrs were 12.26% and 10.33%, respectively.

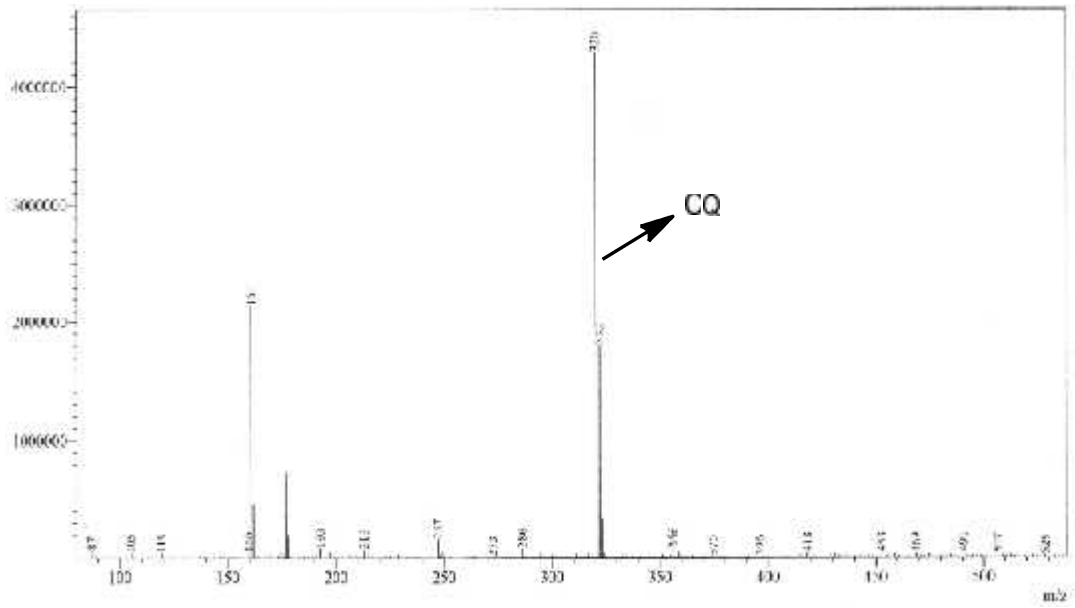


Figure 29: MS spectrum showing chloroquine standard

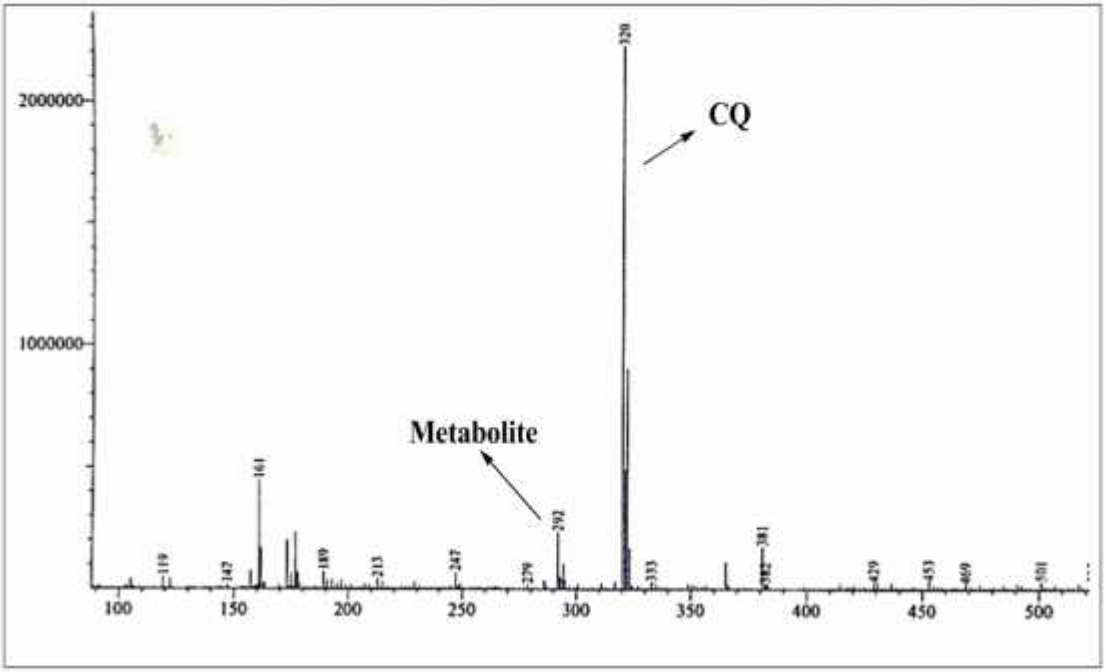


Figure 30: MS spectrum showing chloroquine and N-desethylchloroquine masses at t=2hr using HLM. Similar chromatogram was observed when the experiment was conducted using CYP2C8.

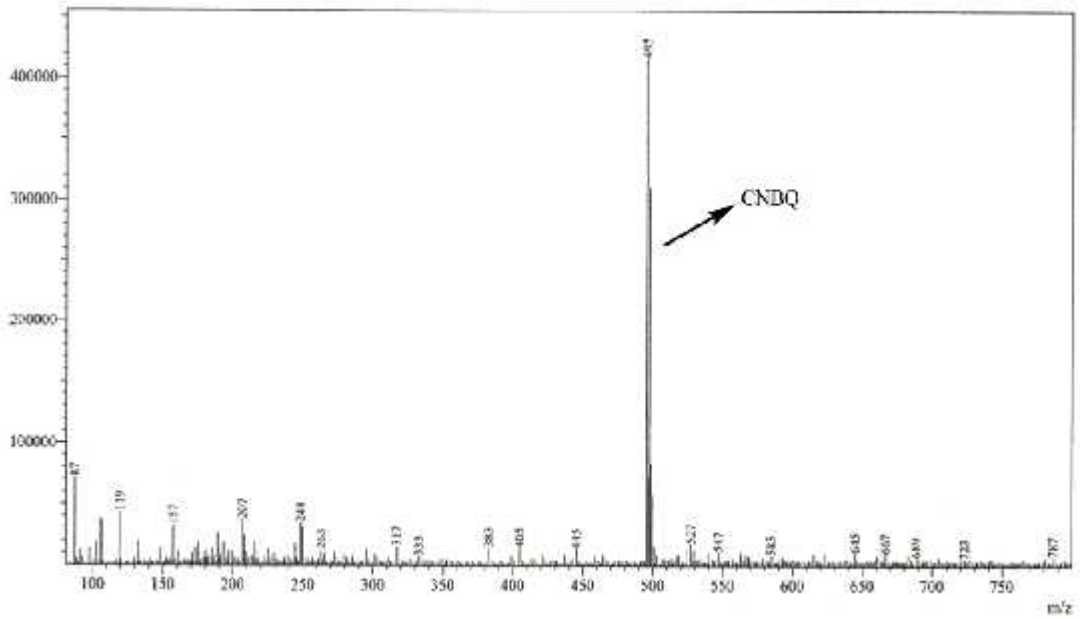


Figure 31: MS spectrum showing drug lead (CNBQ) standard

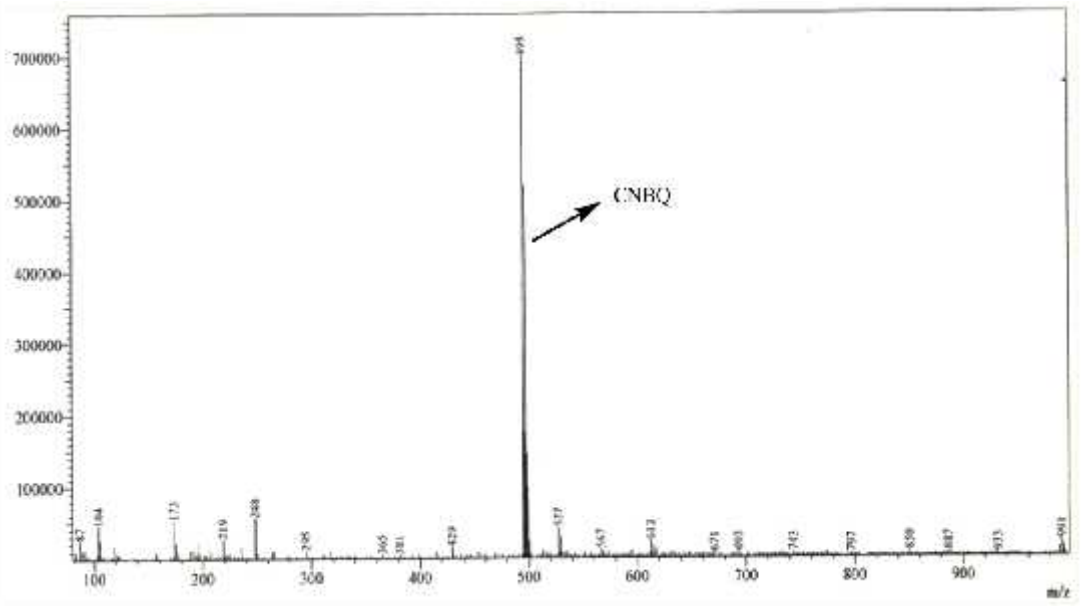


Figure 32: MS spectrum showing drug lead mass at t=2hr using HLM. Similar chromatogram was observed when the experiment was conducted using CYP2C8.

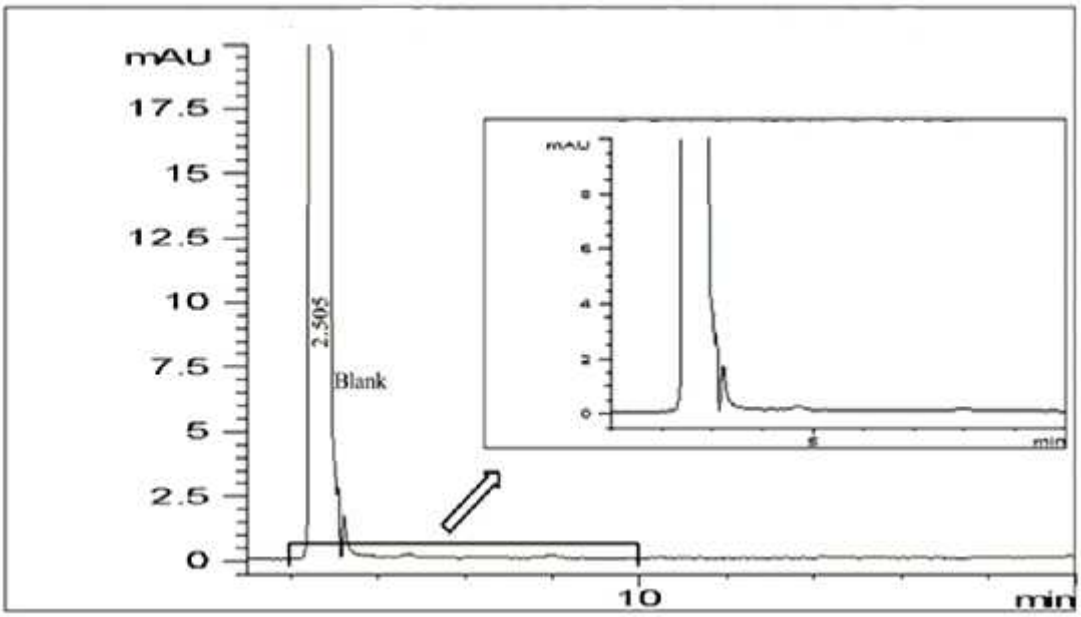


Figure 33: HPLC chromatograms of chloroquine; Blank (NADPH, Enzyme and Buffer)

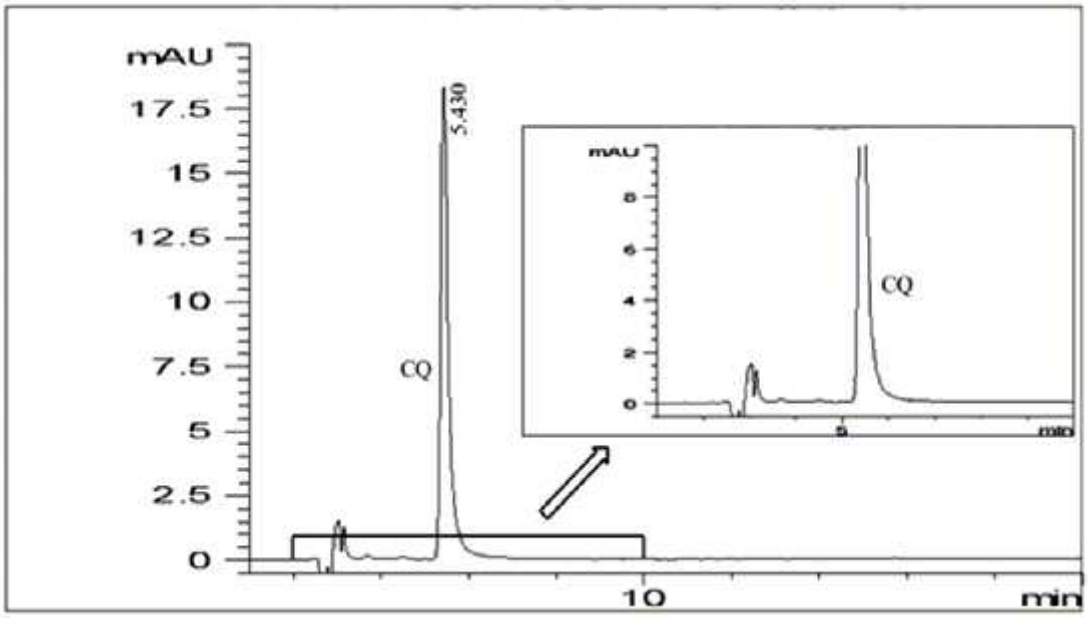


Figure 34: HPLC chromatograms of chloroquine; Control without Enzyme

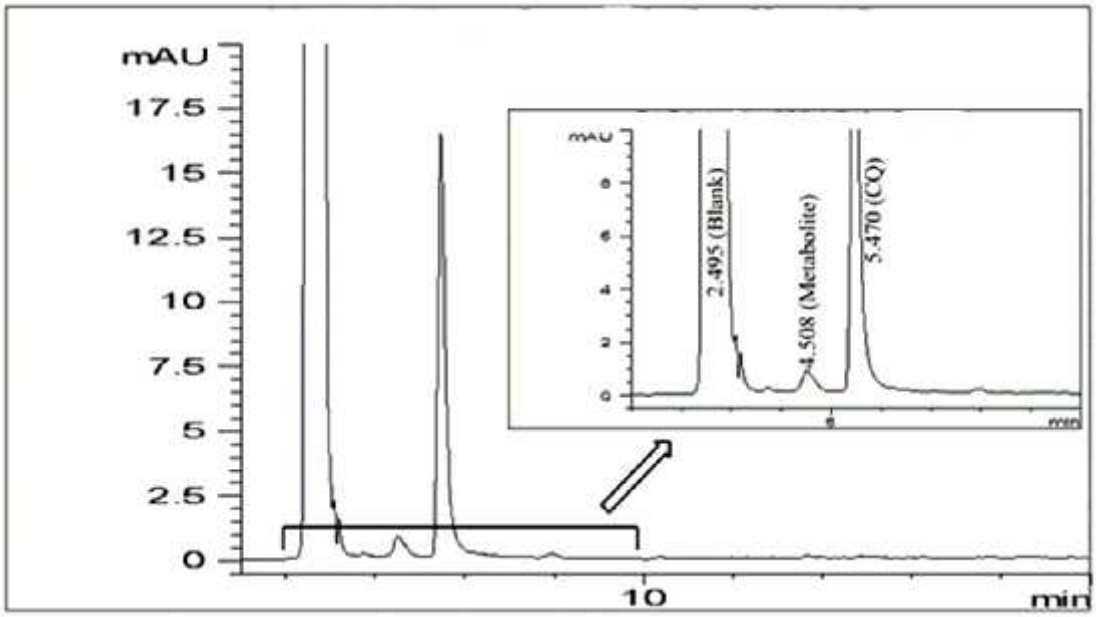


Figure 35: HPLC chromatograms of chloroquine in HLM (t=2hr)

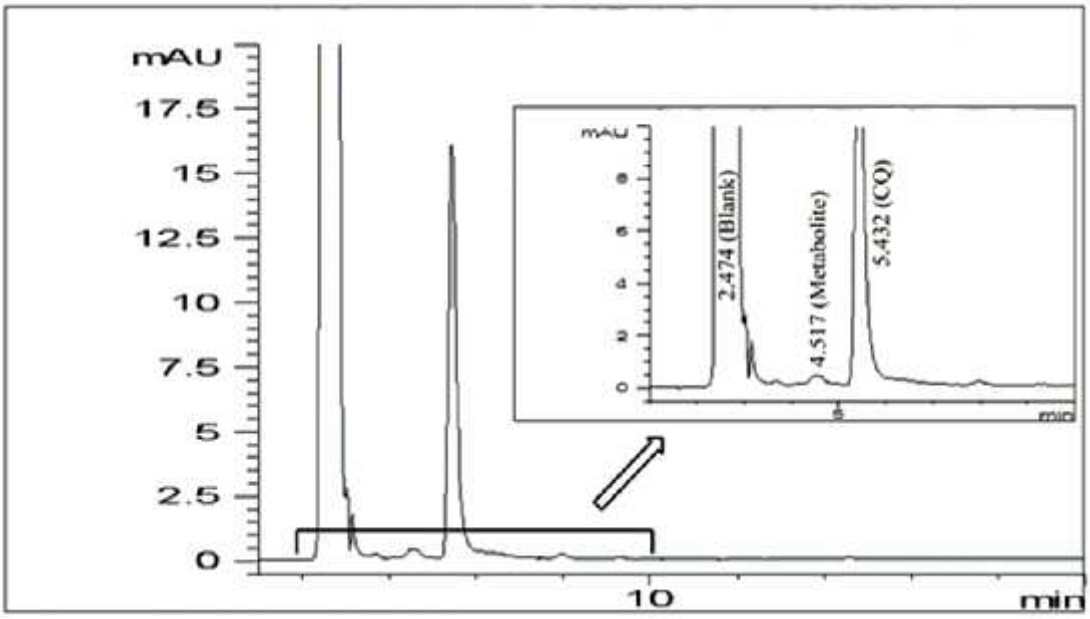


Figure 36: HPLC chromatograms of chloroquine in CYP2C8 (t=2hr)

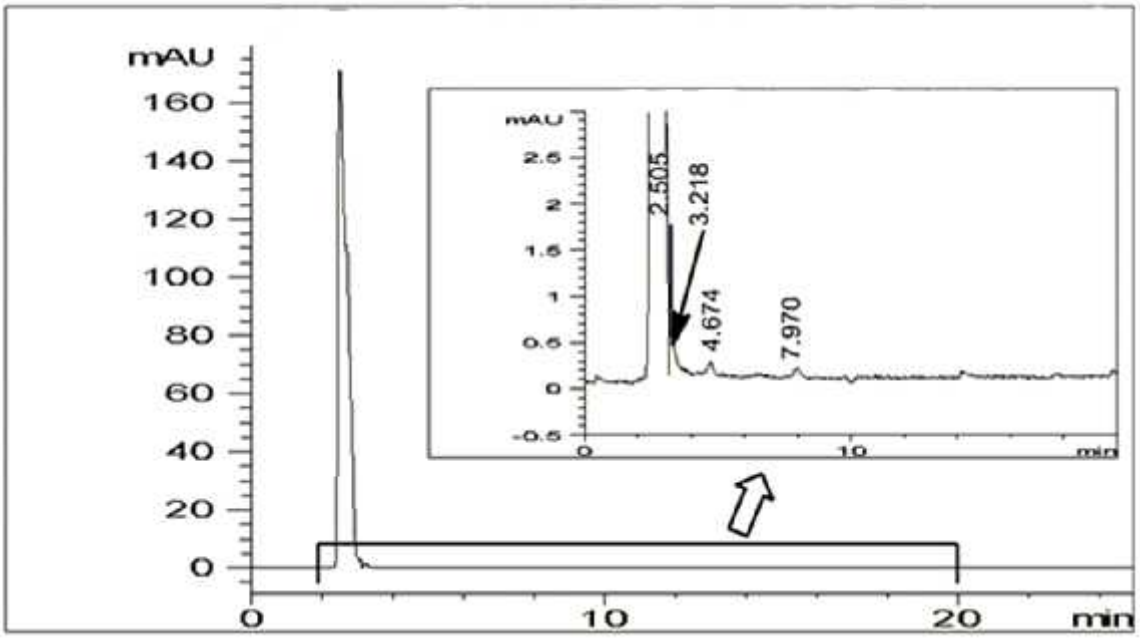


Figure 37: HPLC chromatograms of drug lead CNBQ; Blank (NADPH, Enzyme and Buffer)

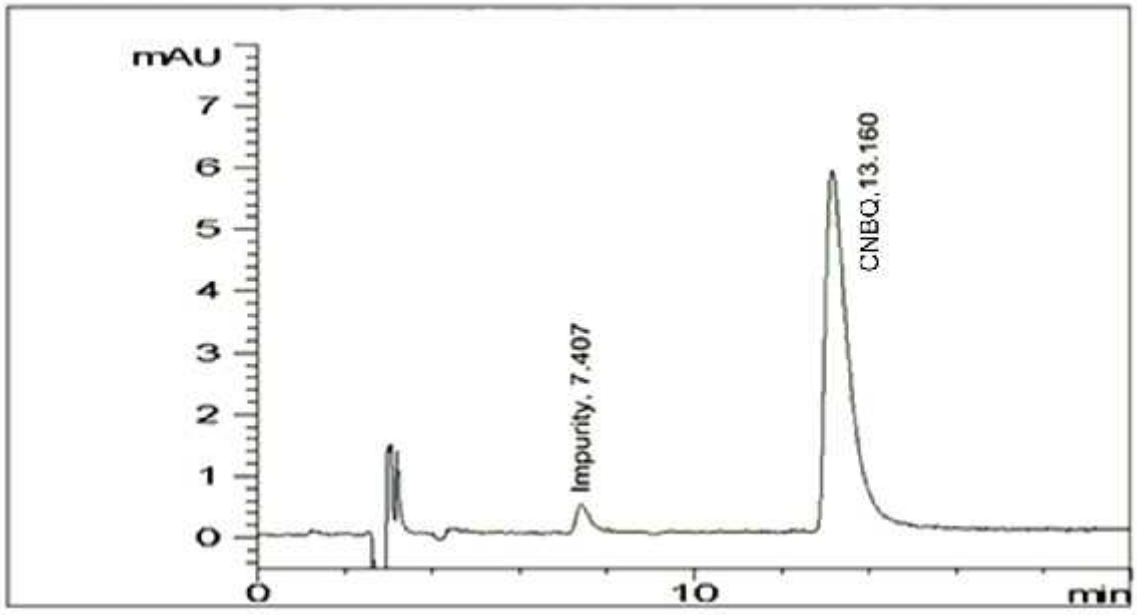


Figure 38: HPLC chromatograms of drug lead CNBQ (Control without Enzyme)

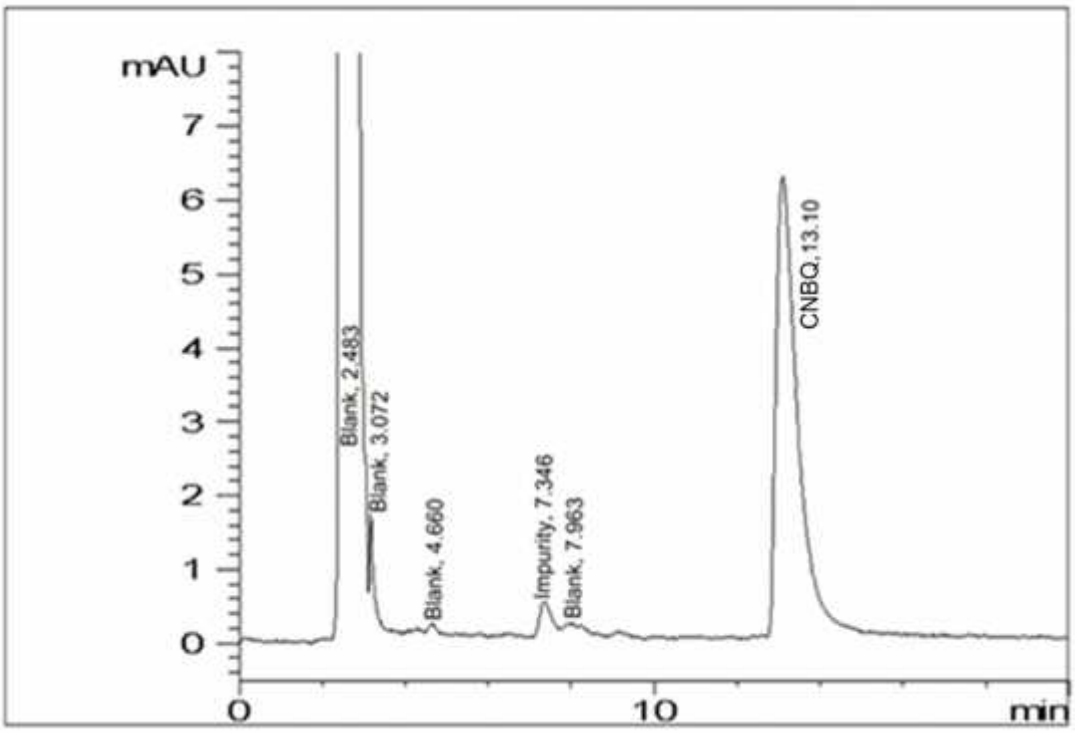


Figure 39: HPLC chromatograms of drug lead CNBQ in HLM (t=2hr)

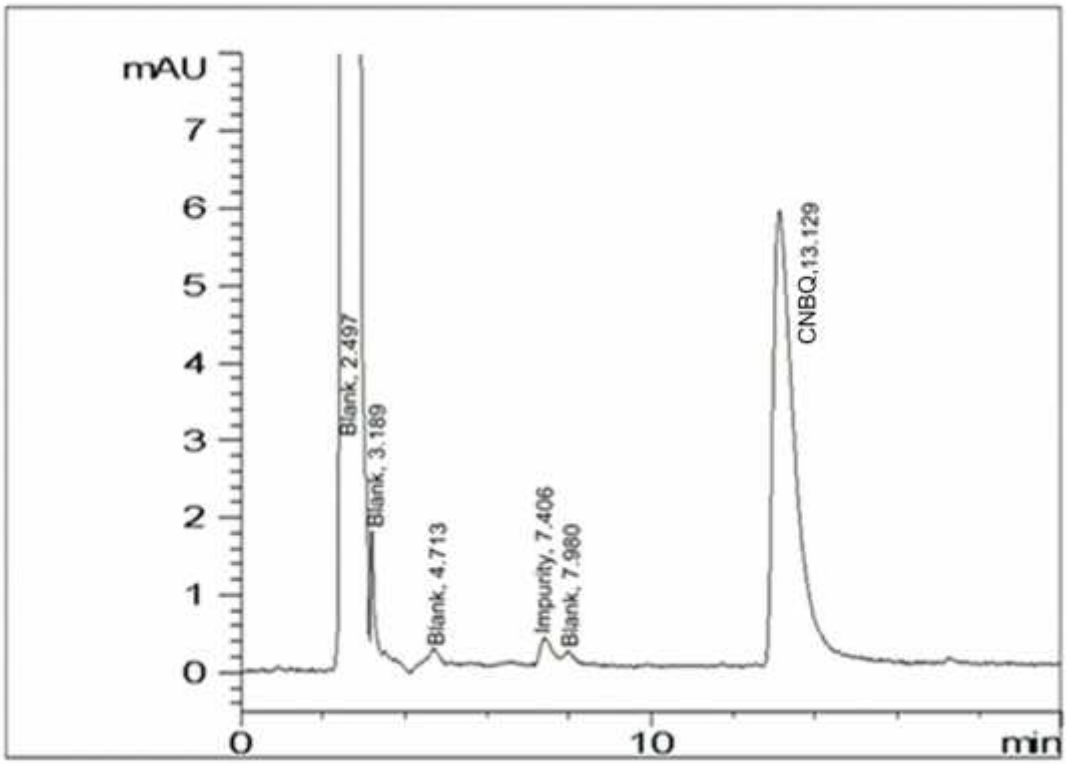


Figure 40: HPLC chromatograms of drug lead CNBQ in CYP2C8 (t=2hr).

The *in vitro* metabolism of the new drug leads compared to the positive control chloroquine in the presence of HLM and cDNA expressing CYP2C8 have been demonstrated in this study.

In the figure 37-40, the HPLC chromatograms clearly showed that drug lead was not metabolized but the appearance of an additional peak before the parent peak in blank and control sample confirms the presence of impurity in drug lead B. The spectrum (Figure. 32) obtained from LC-MS showed the ion peak of the drug lead at time t=2hrs as 495m/z, which confirms its molecular weight. Henceforth there is no metabolism of the drug lead. Nebert and Russell[36] mentioned that there are 270 different CYP gene families, and 18 of those gene families were recorded in mammals that encrypt 57 CYP genes in humans. Out of all CYP enzymes, CYP2C8, 3A4, and 2D6 are involved in Chloroquine metabolism. It has low affinity and high capacity in metabolizing the chloroquine. Further investigations would be conducted utilizing more CYP isozymes at different concentration ranges and time intervals to identify any possible metabolic pathways for these drug lead in near future as a part of our continuous preclinical analysis.

5.5.6 Conclusion

This study demonstrated a convenient method development and also a metabolic stability of the drug leads against most important CYP isozyme that is involved in the metabolism of standard drug chloroquine. According to the results obtained from HPLC and MS data, it was confirmed that both HLM and CYP2C8 has an important role in the metabolism of chloroquine whereas, the drug lead CNBQ was metabolically stable to these enzyme.

5.6 DETERMINATION OF pKa VALUES

5.6.1 Abstract

The purpose of this experiment was to evaluate and compare the pKa values of the poorly water soluble, polyprotic weakly basic drug lead, CNBQ. Three separate methods, pH-metric, UV-metric, and reverse phase-high performance liquid chromatography (RP-HPLC), were employed to determine the pKa values between 2.0-12.0 pH range. The acetate and phosphate buffers, in addition to methanol and acetonitrile as co-solvents and potassium chloride to maintain the ionic strength, were used as appropriate. In UV-metric method, the drug substance is dissolved in aqueous media eliminating any interference of a co-solvent for measuring the pKa. Consequently, the pKa values obtained by the UV-metric method are considered accurate, as opposed to Potentiometric and RP-HPLC methods that require the use of co-solvents. Thus, through the utilization of UV-metric method three pKa values, 5.9, 6.6, and 8.7, were obtained for CNBQ. These studies would be useful to determine the pKa values of the related drug leads under development.

5.6.2 Introduction

The acid dissociation constant (pKa) is the pH at which concentrations of ionized and unionized forms of drugs are equal. It is an essential parameter in drug discovery, particularly in physiological systems where ionization state will affect the rate at which the compound is able to diffuse across membranes including blood-brain barrier.[37] Unless the drug is given intravenously, the drug must pass through several different semipermeable membranes before it reaches the systemic circulation and later in the site of action. The semipermeable cell membranes, due to their inherent hydrophobicity, selectively inhibit the passage of drug molecules. The simplest route for a drug to enter the systemic circulation is by passive diffusion through the semipermeable cell membranes from an area of high concentration to an area of low concentration. This requires the ingenuity from drug developers to design drug

substances that will overcome these obstacles and allow the proper passage through the semipermeable membranes.

Most drugs are weak organic acids or bases existing in ionized and unionized forms in aqueous solutions. The unionized forms of drugs are usually more lipophilic and thus readily diffuse across cell membranes. It is in this instance that pKa and bioavailability correlate. When the pH is lower than the pKa, the unionized form of a weak acid predominates, and vice versa for a weak base.[38] Proceeding with this concept, different pH in the body allow for the alteration of solubility, dissociation, and coincident absorption of the drug substance[39] by shifting the concentration of the unionized and ionized forms of drug substances, therefore, validating the importance of determining the pKa of a drug.

The antimalarial drug lead cyclen bisquinoline (CNBQ; Figure 2) demonstrated a potent *in vitro* anti-malarial activity against chloroquine-sensitive and chloroquine-resistant as well as mefloquine-resistant strains of *Plasmodium falciparum*. As malaria is caused by invasion of malaria parasites in the blood, it is important that the drug substance intended to treat it is able to enter the systemic circulation. Therefore, evaluation of pKa values is imperative in the early stage of drug discovery and development, because, as previously mentioned, pKa values of the drug substance and its bioavailability are correlated. There are several different methods that can be employed to determine the pKa values of the drugs, such as: pH-metric, UV-metric, NMR, solubility, capillary electrophoresis, HPLC, conductometry, voltammetry, calorimetry, fluorimetry, polarimetry, kinetic, and computational methods.[40] It is challenging to find a single method that will not only measure any and all pKa values, but also be reliable in its findings in just one attempt due to the vast amount of variability in drug structures. This paper will focus on the following three methods: pH-metric, UV-metric, and the RP-HPLC methods. Many of other methods previously mentioned require a substantial amount of instrumentation, test material and/or time, making them unsuitable or not feasible for implementation in this research lab.

5.6.3 Materials and Methods

5.6.3.1 Materials:

CNBQ (Figure 2) was synthesized in our laboratory and the purity checked by HPLC. Chloroquine diphosphate was purchased from Pfaltz & Bauer, USA. The solvents and reagents used were as follows: acetonitrile, methanol, sodium hydroxide, potassium hydroxide, hydrochloric acid, potassium chloride, ammonium acetate, and sodium phosphate dibasic anhydrous. Each solvent and reagent used was HPLC and analytical grade and was purchased from Fisher Scientific. Deionized water used to prepare the solutions and mobile phase was further purified by filtration and degassing.

5.6.3.2 Methods:

5.6.3.2.1 pH-metric method (Potentiometric titration):

The pH-metric titration was performed using two different instruments, highly sensitive fully automated Sirius T3 in Sirius laboratory and semi-automated Metrohm in our laboratory. In Sirius laboratory, 0.01 M phosphate buffer was used to prepare the drug solution, and pH of the buffer were accurately adjusted from pH 2.0 to 12.0 with 0.2 interval using 0.5M HCl and 0.5M KOH titrants as appropriate. The pH electrode of the potentiometer was calibrated using standard buffers at pHs of 4.0, 7.0 and 10.0. 0.1M hydrochloric acid was used to prepare the drug solution, and pHs of the buffer were adjusted from pH 2.0 to 12.0 using 0.1 M NaOH/0.1 M KOH titrants as appropriate. In both cases, the ionic strength of the solution was maintained using 0.15M potassium chloride solution and methanol was used as a co-solvent. The solution temperature was set at 25°C and nitrogen purging was performed to displace the dissolved gases from titrating solutions in both experiments.

In the pH-metric titration, a known volume of reagent is added in a step wise mode to the analyte. The change in the measure of potential is determined by the use of two electrodes, an indicator and a reference electrode.[41] The changes in potential vs. pH are graphed; subsequently producing a sigmoidal curve allowing the determination of the pKa of the compound. The pKa value is the pH at half-neutralization point, which

represents the center point on the ascending portion of the sigmoidal curve, and was integrated by automated software.

5.6.3.2.2 UV-metric method (Spectrophotometric determination)

Spectrophotometric determination of pKa was performed using two different instruments, highly sensitive fully automated Sirius T3 in Sirius laboratory and semi-automated NanoDrop 2000c Spectrophotometer in our laboratory. With the implementation of Sirius T3 instrument, the sample was subsequently titrated in a UV-metric triple titration from pH 2.0 - 12.0 at concentrations of 11-16μM under aqueous conditions (0.01M Phosphate Buffer). The ionic strength of the solution was maintained using 0.15M potassium chloride solution. The buffer allowed for controlled pH as it was adjusted from pH 2.0 to 12.0 using 0.5M HCl and 0.5M KOH titrants. The pKa values were determined using dip-probe absorption spectroscopy (D-PAS) technique. In the D-PAS technique, a fiber optics dip-probe, a UV-light source (Deuterium Light), and a photodiode array detector were used in conjunction with a titrator to capture the spectral changes which arise during the course of titration. Software set up all experimental data in an absorbance matrix based on Beer's law (Absorbance = Concentration x Extinction Coefficient), and target factor analysis (TFA, at the rate of change at which the compound's UV absorbance was the strongest) detected the corresponding pKa values from the absorbance matrix.[42]

The second experiment utilized the NanoDrop Spectrophotometer instrument and 1 cm^2 UV-cell. UV spectra were taken throughout the course of titration with an approximate interval of 0.2 pH unit, between pH 3.0-11.0 at concentrations of 0.05mM under aqueous conditions (0.01M Phosphate Buffer). The ionic strength of the solution was maintained using 0.15M potassium chloride solution. The pH of the buffer was adjusted by addition of 0.5M HCl and 0.5M KOH solution using ACCUMET pH meter. The corresponding pKa values were determined by visual evaluation based on changes in the compound's UV spectra pattern with respect to pH. The pKa value corresponds to the pH at which the rate of change of the UV spectra of the compound is the strongest.

5.6.3.2.3 RP-HPLC method:

To determine the pKa using RP-HPLC, 0.084 mg/ml solution of CNBQ as a test sample, and 0.032 mg/ml solution of chloroquine diphosphate as a reference sample were prepared in a 50:50 solution of acetonitrile and water. The solutions were then injected using the following chromatographic conditions to determine the retention time in different pH values of the mobile phase. The pKa values were determined from the first derivative curve of the retention time vs. pH values.

Chromatographic separation of CNBQ and chloroquine were successfully achieved on a Waters X-Bridge C-18 column (4.6mm x 250mm, 5.0µm particle size, part no. 186003117) purchased from Waters Corporation in an isocratic separation mode with a mobile phase consisting of 50% of acetonitrile and 50% of 0.002M ammonium acetate. The pH of the mobile phase was set within the range of 7.0-12.0 with an interval of 0.2 with 0.1N HCl and 0.1N NaOH. The flow rate was maintained at 1.0 ml/min, the column oven temperature was maintained at 25°C, the injection volume was set at 1µL, and the effluent was monitored at 254nm.

5.6.4 Analytical Method Validation Report

pKa values (Table 13) obtained by UV-metric method (Manual) and RP-HPLC method were verified by a comparative study with the results obtained by automated UV-metric and pH-metric method. pKa values obtained by manual UV-metric method, conducted in our lab, plotted against those obtained by fully automated Sirius T3 instrument, it exhibited a straight line with R^2 value of 0.993 (Figure 46), signifying the accuracy and validity of the results. To validate the pKa values obtained using in-house RP-HPLC method, chloroquine was used as reference.

5.6.5 Results and Discussion

As mentioned previously, the pKa values of CNBQ were determined using the following three methods: pH-metric, UV-metric and RP-HPLC. Considering the variability in the results obtained from the three aforementioned methods, three pKa

values: 5.9, 6.6, and 8.7 were estimated for the compound. Table 13 represents the pKa values observed and calculated from these methods.

Table 13: pKa values of CNBQ by different methods.

pKa Values	**pK_{a1}**	**pK_{a2}**	**pK_{a3}**
UV-metric method (Automated)	5.87±0.01	6.60±0.01	8.73±0.01
pH-metric method (Automated)	5.80±0.01	6.35±0.11	8.43±0.03
UV-metric method (Manual)	5.99	6.81	8.67
RP-HPLC method	---	---	8.8

The Potentiometric determination of pKa is a relatively simple, which can be used for any ionizable compound that does not require presence of chromophore groups for pKa determination.[43] pKa determination by this method is the most economical method, in regard to the short duration of experiment and the ease of reproducibility if carried out correctly. Limitations of this method has been the need for a higher amount of drug substance in order to achieve an accurate result and necessity for the use of co-solvents for poorly water soluble basic compound to prevent precipitation of the drug at higher pH values. CNBQ, being insoluble at higher pH, requires extrapolation using methanol as a co-solvent. Use of a co-solvent impacts the pKa values,[44] resulting in a higher level of variability than normally observed.

Table 14 represents the data obtained by pH-metric method. pKa values of CNBQ was determined using Yasuda-Shedlovsky standard extrapolation method[45] as shown in Figure 41, in which X-axis plots the inverse of the dielectric constant of the water-solvent mixture at the experimental percentage of solvent and Y-axis plots the psKa + log[H_2O]; where, psKa is cosolvent dissociation constants, and [H2O] is the molar water concentration of the given solvent mixture. The extrapolated value represents the pKa value at 100% water concentration (0% co-solvent). Sirus T3 software could conveniently calculate the extrapolated pKa values in water using this method. The pKa values of CNBQ were determined by this method to be 5.8, 6.4 and 8.4 with a R^2 value of 0.99. Another attempt was made to determine the pKa value of CNBQ using a Metrohm potentiometer in our laboratory. In this experiment, methanol and

acetonitrile were used as co-solvents. Although the experiment was performed in a controlled setting, it did not produce any reproducible pKa value due to poor solubility of the basic compound, and thus precipitation of the samples in the solution as pH is increased, and lack of sensitivity of the instrument. In general, a higher quantity of the sample is required to determine the pKa using an ordinary potentiometer that causes precipitation of the sample and thus inaccurate results. However, due to recent advances in technology (Sirius T3, as discussed above), pKa values can now be determined in a more economical approach using smaller amounts of sample, which is very important in the early stage of drug development.[46]

Table 14: pKa values of CNBQ by pH-metric method using Sirius T3

pKa Values	%Methanol	Dielectric Constant	[H2O]	psK_{a1}	psK_{a2}	psK_{a3}
Experiment 1	29.2	65.9	36.4	5.4	6.0	8.3
Experiment 2	38.9	61.5	30.6	5.2	5.9	8.2
Experiment 3	49.5	56.6	24.7	4.9	5.6	8.2

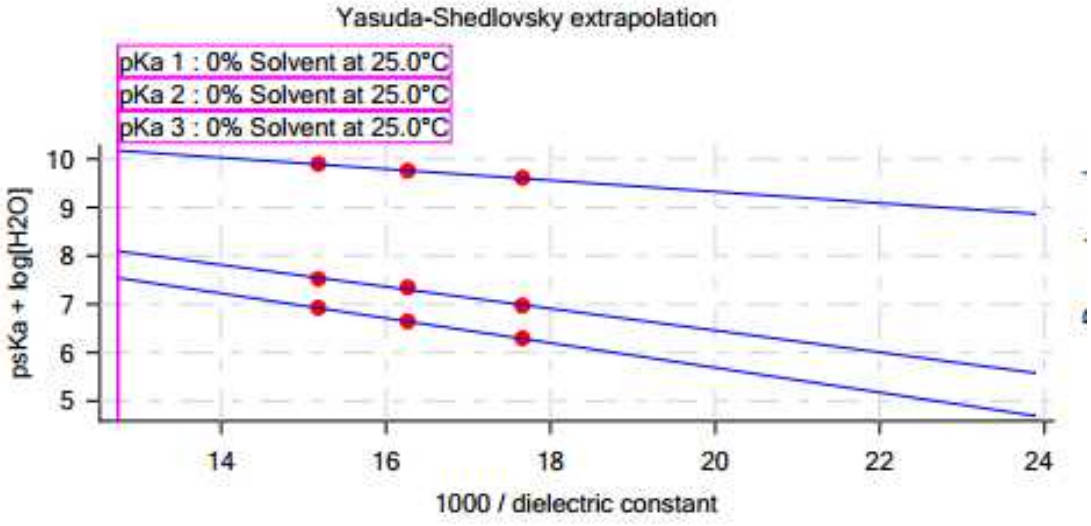


Figure 41. Yasuda-Shedlovsky standard extrapolation in pH-metric method by Sirius T3.

RP-HPLC method was developed to overcome the challenges posed by the properties of current drugs with low solubility. This method is attractive due to its simplicity and its ability to use a variety of isocratic HPLC systems with the use of less drug material.[47] pKa is determined by analyzing the change in retention time of an analyte vs. the pH of the mobile phase of the respective retention time. Utilization of RP-HPLC method has proven to be successful in determining pKa values that resemble literature pKa values.[48,49] The fundamental principal of this method is the variation of retention time based on the pH of the mobile phase. However, obtaining a sharp peak shape and accurate retention time at each point using an isocratic mobile phase in the pH range of 2.0 to 12.0 is quite difficult when dealing with polyprotic basic compound having multiple pKa values. Improvement of peak shape can be accomplished by addition of an organic modifier and an ion pairing reagent to the mobile phase, but addition of these reagents will result in inaccurate pKa values.[47] CNBQ is a polyprotic basic drug and shows a broader peak shape in the lower pH ranges, which are not reproducible. It was revealed in this experiment that good peak shape for CNBQ can be obtained within the pH range of 7.0 to 12.0 as shown in Figure 42. Thus, the pKa determination was performed in the pH range of 7.0 to 12.0, using acetonitrile as an organic phase and acetate buffer as the aqueous phase. Because of the structural similarities between chloroquine and CNBQ, chloroquine was used as a reference throughout the entire experiment. The pKa values for both compounds were determined from the first derivative curve of the retention time vs. pH values (Table 15 & Figure 43). The pKa values for chloroquine obtained by RP-HPLC method were 8.4 and 10.2 (compared to the literature values of 8.37 and 10.49, respectively).[45] In regards to CNBQ, RP-HPLC method only produced one pKa value of 8.8, which proves the difficultly in determining multiple pKa values using RP-HPLC method.

The UV-metric method measures absorption, emission, or scattering of electromagnetic radiation with respect to changes in pH, leading to changes in the UV spectra of the molecule.[50] These changes in the multi-wavelength UV spectra can be observed, if the disposition of electrons in chromophore containing conjugated double bonds, carbonyl groups, and other UV absorbing groups change with the molecule's ionization state. There are several methods to calculate the pKa values from UV

absorbance data vs. pH.[46,50-52] There are two disadvantages of the UV-metric method. First, if the compound is too basic it may precipitate out; however, this can be overcome if the experiment is performed in the presence of a co-solvent, but would require extrapolation to obtain the final results. Second, if the compound possesses no pH-active chromophore, then the UV-metric method cannot be applied making this the major disadvantage. If the sample has a UV response and exhibits no/less precipitation, then it is possible to conduct the experiment under aqueous conditions using only small amount of the compound.[46] This results in pKa values which are considered more accurate, because there is no interference of a co-solvent and no extrapolation is needed to obtain the final results. In this experiment using automated software of Sirius T3 instrument, TFA detected the corresponding pKa values at which the rate of change in the compound's UV absorbance is the strongest. CNBQ exists in four different species; BH3, BH2, BH, and B at depending on the pH of the medium. Figure 44 represents the spectra for four different species of CNBQ obtained by Sirius T3 instrument. Utilizing this method, three pKa values of 5.87, 6.60, and 8.73, were observed from the spectroscopic data. Table 16 represents the data obtained from automated UV-metric method.

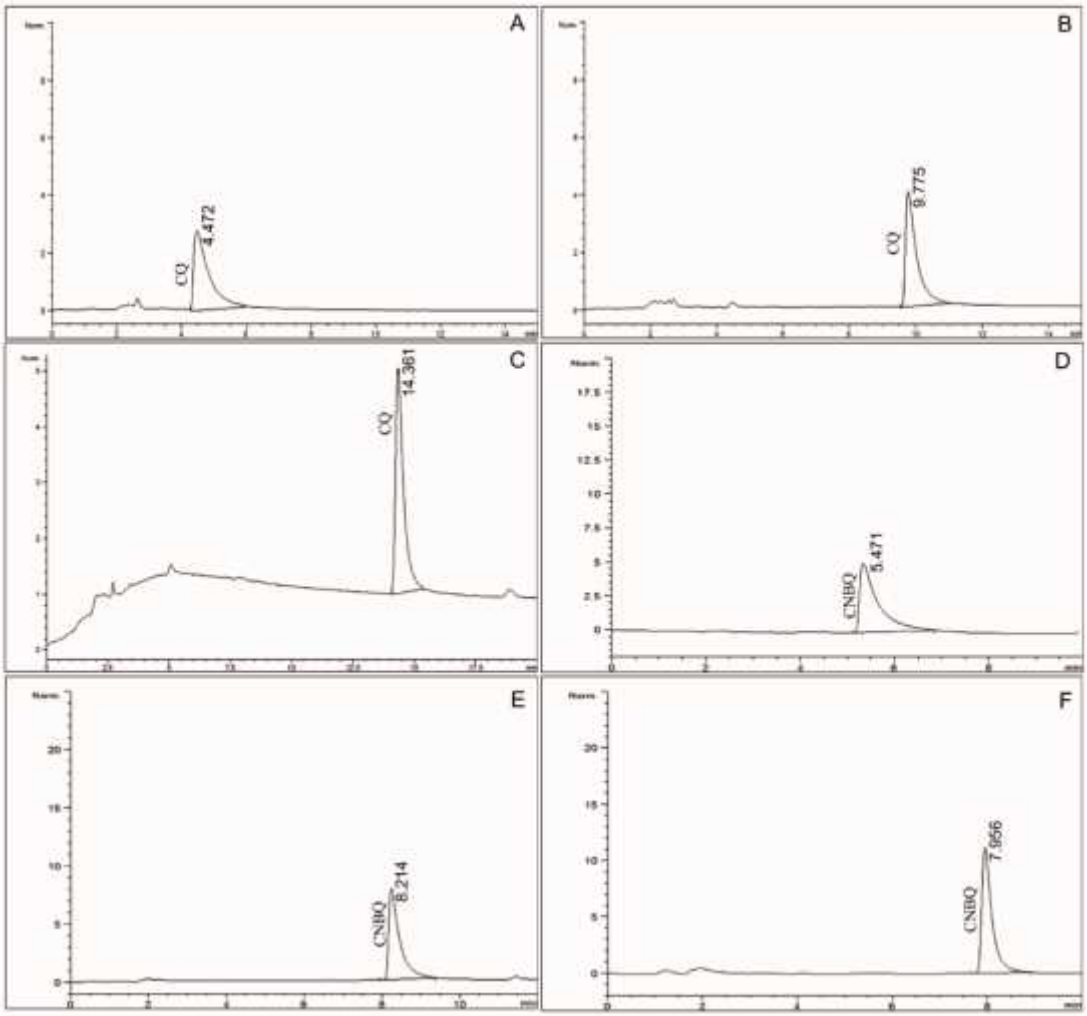


Figure 42. HPLC Chromatograms; A: CQ at pH 7.3, B: CQ at pH 9.5, C: CQ at pH 11.1, D: CNBQ at pH 7.5, E: CNBQ at pH 9.4, F: CNBQ at pH 11.1.

Table 15: Retention time (RT) and pH of the mobile phase.

Chloroquine			CNBQ		
RT	**pH**	**ΔRT/ΔpH**	**RT**	**pH**	**ΔRT/ΔpH**
4.472	7.31	---	5.338	6.97	---
4.460	7.50	0.179	5.471	7.49	0.256
4.528	7.88	0.311	5.647	7.64	1.173
4.584	8.06	0.926	6.000	7.88	1.471
4.797	8.29	1.931	6.341	8.13	1.364
5.048	**8.42**	**3.700**	6.774	8.41	1.546
5.455	8.53	3.231	7.094	8.60	1.684
5.875	8.66	3.604	7.414	8.75	2.133
7.533	9.12	5.468	**7.734**	**8.84**	**3.556**
9.775	9.53	5.715	8.023	9.14	0.963
12.518	10.01	3.894	8.214	9.42	0.682
13.180	**10.18**	**6.417**	8.278	9.64	0.291
13.950	10.30	1.477	8.286	9.74	0.080
14.334	10.56	0.055	8.284	9.97	-0.009
14.361	11.05	-0.178	7.956	11.07	-0.298

Figure 45 represents the spectra of different species of CNBQ obtained from NanoDrop 2000c Spectrophotometer at different pHs. In this figure, pKa_1, pKa_2 and pKa_3 represent the strongest changes of spectrum from one species to another species. The corresponding pKa values of 5.95, 6.83, and 8.67, were determined by visual evaluation based on changes in the compound's UV spectra pattern with respect to pH (Table 17 & Figure 45). When the pKa values obtained by this manual UV-metric method, conducted in our lab, plotted against those obtained by fully automated Sirius T3 instrument, it exhibited a straight line with R^2 value of 0.993 (Figure 46), signifying the accuracy and validity of the results. Figure 47 represents the four different spectra for the species of CNBQ obtained by NanoDrop 2000c Spectrophotometer.

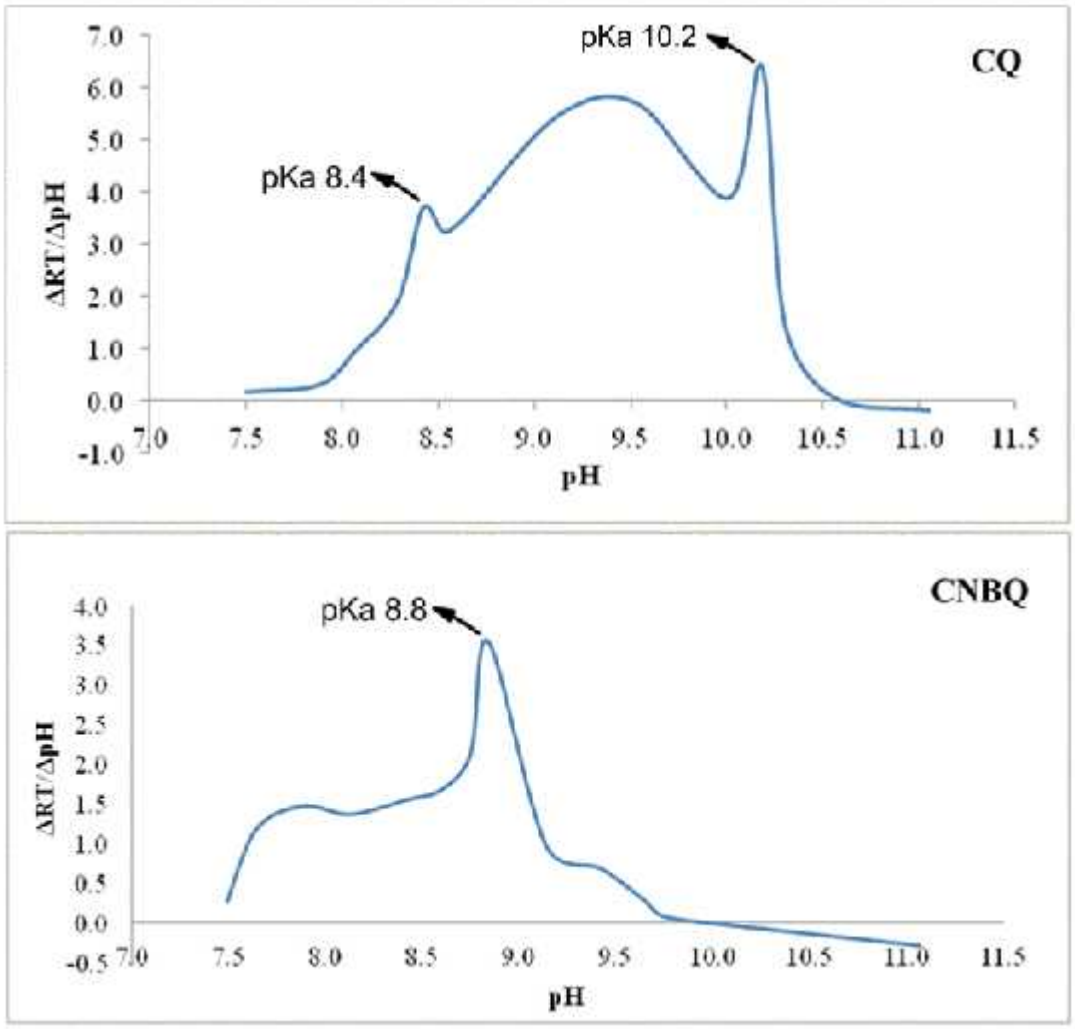


Figure 43. First derivative curves for pKa determination; Top, chloroquine, bottom, CNBQ.

Table 16: pKa values of CNBQ by UV-metric method using Sirius T3.

pKa Values	pK_{a1}	pK_{a2}	pK_{a3}
Experiment 1	5.87	6.59	8.73
Experiment 2	5.89	6.61	8.73
Experiment 3	5.86	6.60	8.73
Average	5.87	6.60	8.73
Std Dev	0.012	0.007	0.004

Table 17: pKa values of CNBQ by UV-metric method using NanoDrop spectrophotometer.

pKa Values	pK_{a1}	pK_{a2}	pK_{a3}
pH of the two consecutive	5.85	6.74	8.56
Spectra	6.05	6.91	8.77
Average	5.95	6.83	8.67

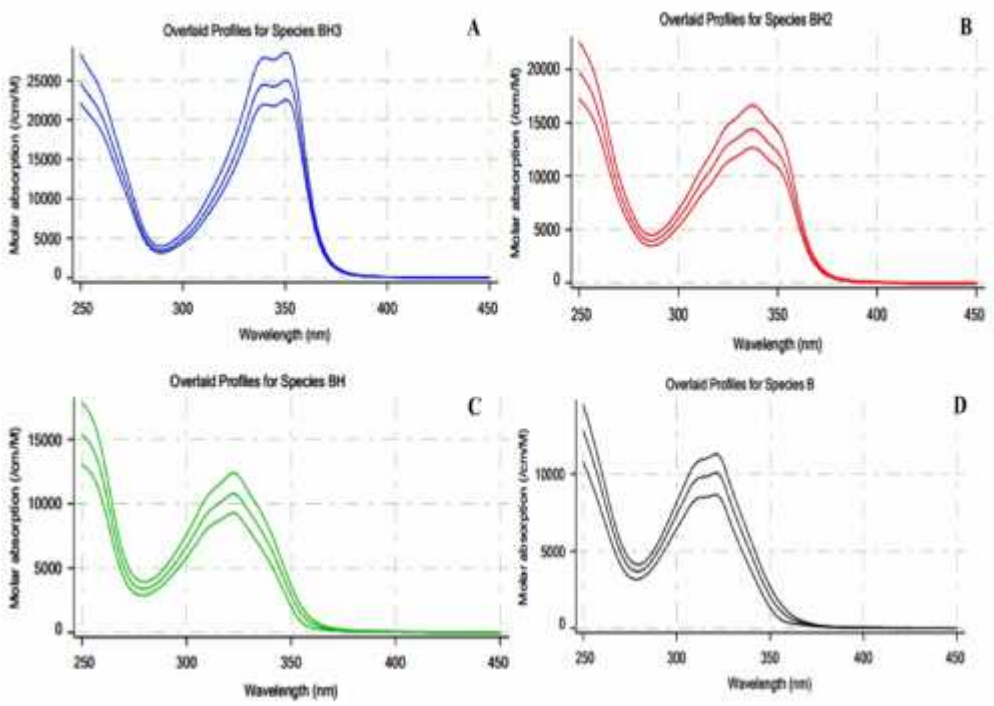


Figure 44. Spectra of different Species of CNBQ obtained from Sirius T3; A: BH3, B: BH2, C: BH and D: B.

Figure 9 represents the distribution of species of CNBQ in different pH conditions by Henderson–Hasselbalch equation. From the species distribution it is clearly shown that the lone pairs of electrons of nitrogen atoms in the aliphatic and aromatic ring of CNBQ will fully accept three protons (BH3 Species) at lower pH (approximately pH 4.0). CNBQ will be partially ionized at an approximate pH of 6.0 (BH2 Species) and at an approximate pH of 8.0 (BH Species), accepting two and one proton(s), respectively. CNBQ will be completely unionized (B Species) at higher pH (approximately pH 10.0).

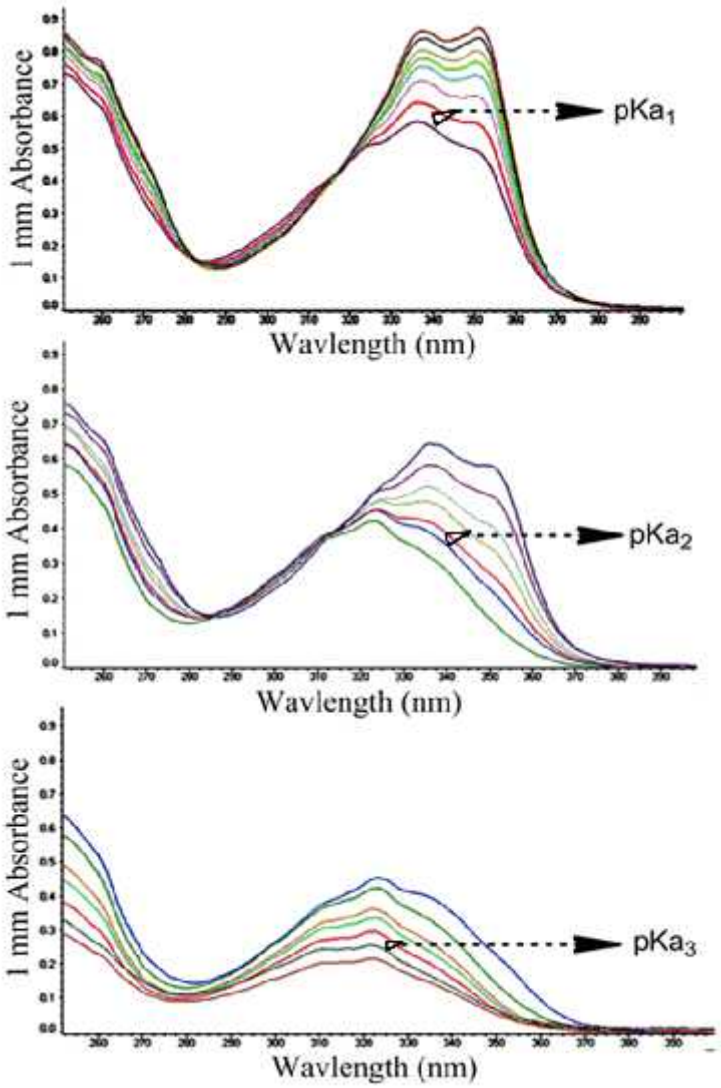


Figure 45. Spectra of different Species of CNBQ obtained from NanoDrop Spectrophotometer at changing pHs.

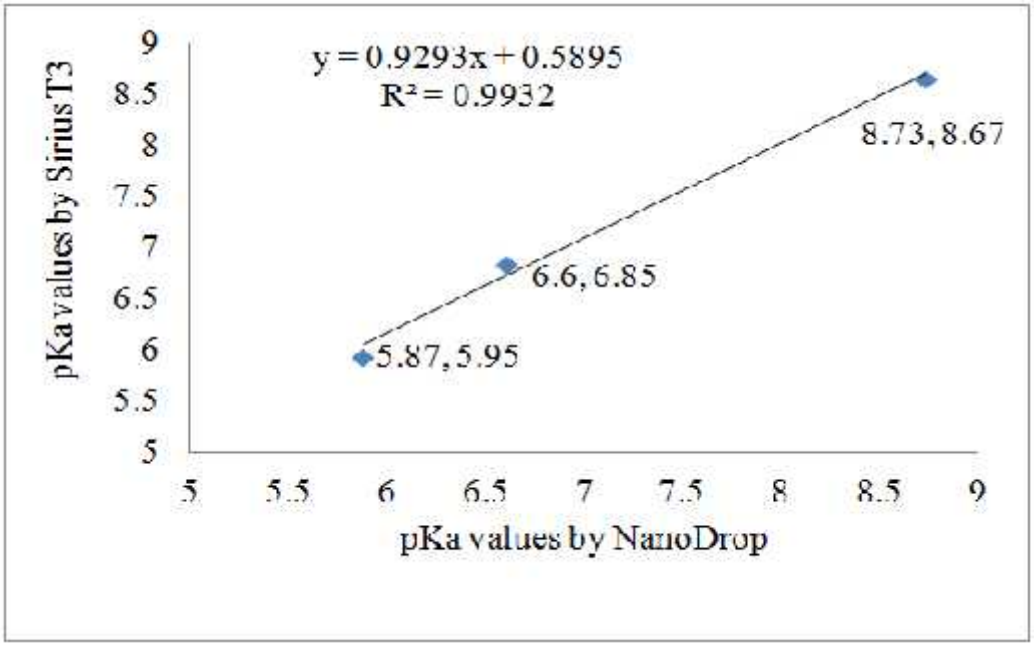


Figure 46. A plot of the pKa values obtained using NanoDrop spectrophotometer against those measured by Sirius T3.

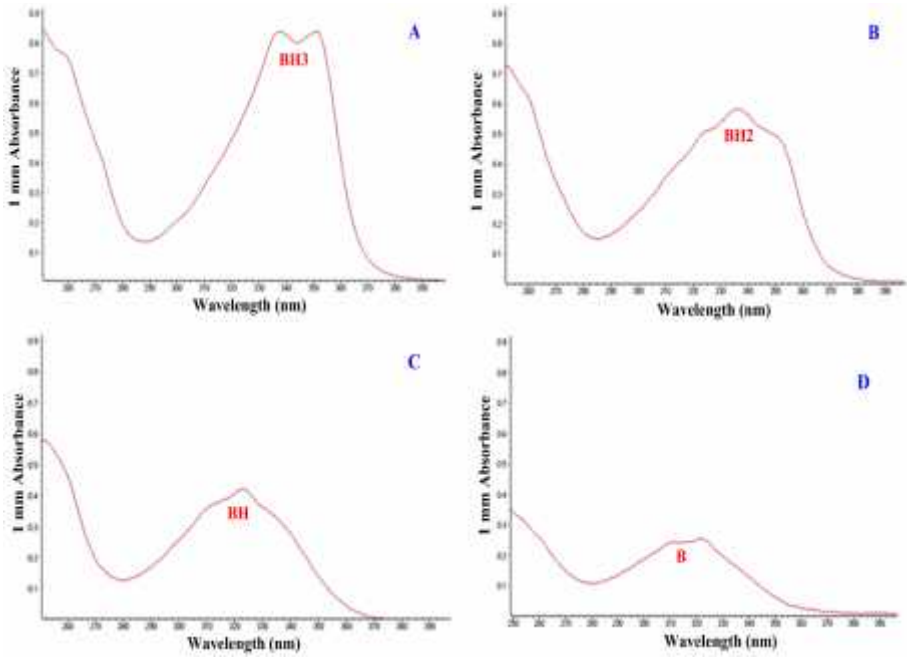


Figure 47. Spectra of different Species of CNBQ obtained from NanoDrop Spectrophotometer; A: BH3, B: BH2, C: BH and D: B.

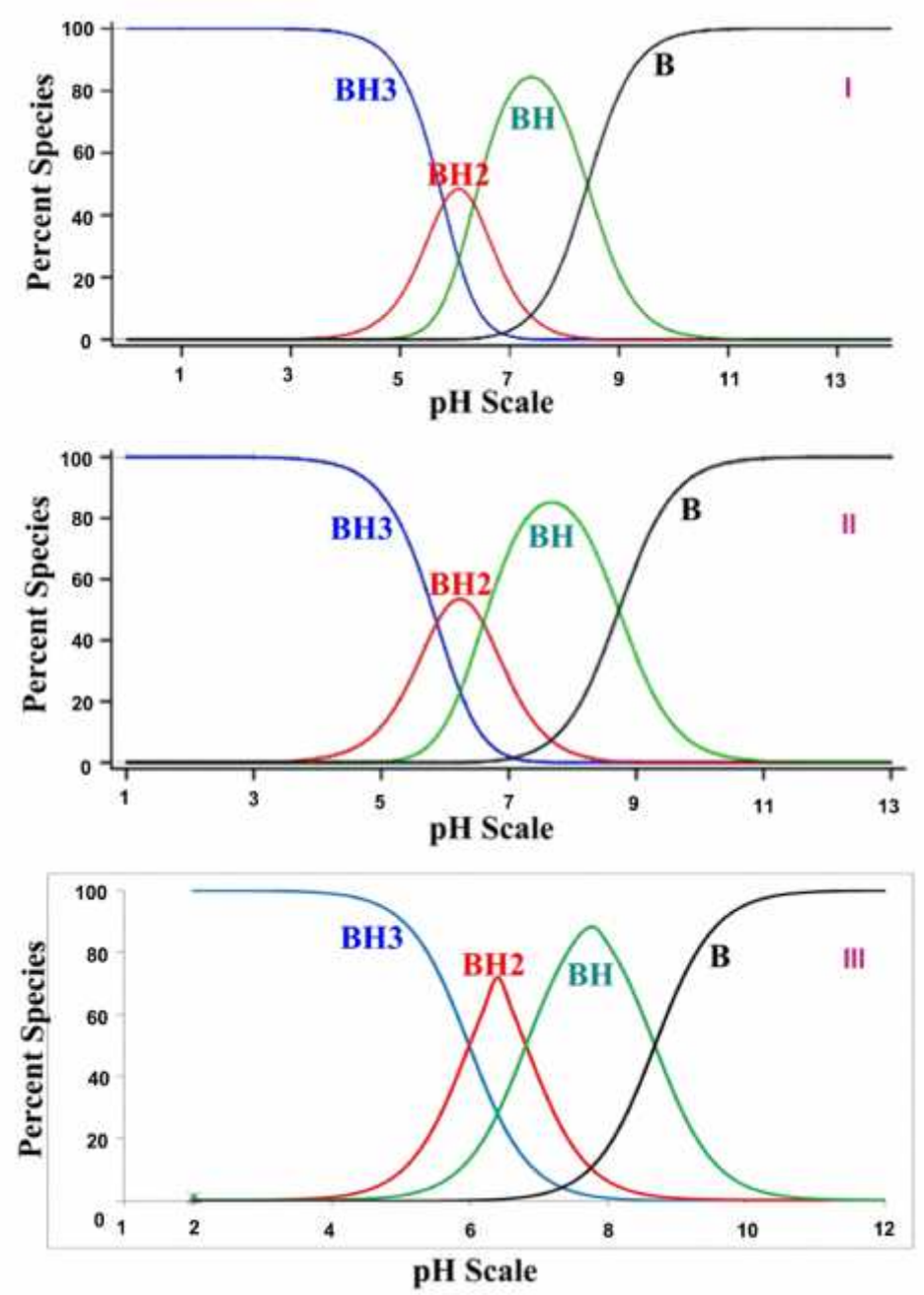


Figure 48. Distribution of species of CNBQ; I: pH-metric method (Sirius T3*)*, II: UV-metric method (Sirius T3*),* III: UV-metric method (NanoDrop*)*.

To validate the pKa values obtained using in-house RP-HPLC method, chloroquine was used as a reference. The pH-metric method and the D-PAS technique for UV-metric method has already been validated using several compounds by Sirius

Analytical Ltd.[42,46] Therefore, UV-metric method using visual inspection was validated by comparing the results obtained at both laboratories (Figure 46). The pKa values obtained from both methods were within ±0.2 with a R^2 value of 0.99. These minor variations are due to changes in instruments, differences in analytical reagents, and different analyst. Since the compound has excellent UV signal and the analysis was performed under aqueous conditions, it did not require an extrapolation. Hence, UV-metric method stands out as an effective method for the determination of multiple pKa values of a poorly water-soluble, polyprotic basic compound having good UV signal.

5.6.6 Conclusion

From the pKa values obtained, it is apparent that CNBQ is a polyprotic basic drug in which the lone pairs of electrons on nitrogen atoms in aliphatic and aromatic ring will accept three protons in different physiological pH conditions. It is imperative that the method chosen is the most accurate method in determining these pKa values. Considering all the methods discussed, UV-metric method is the most suitable in determining the pKa values for the drug lead CNBQ due to its bacisity, inherent insolubility, and multiple pKa values. The RP-HPLC method possesses limitations, in regards to the pH range (pH 7.0 to 12.0 as observed in our experiment), of the mobile phase, making it incapable of determining pKa values that fall outside the range. Use of co-solvents complicates results obtained from both RP-HPLC and pH-metric methods, as the co-solvent will produce a pKa value that is not accurate compared to the actual value. Furthermore, results obtained from pH-metric and RP-HPLC methods require extrapolation making them variable and less accurate than those obtained from the UV-metric method, which does not require co-solvents or extrapolation. In conclusion, RP-HPLC and pH-metric methods are not suitable methods for determining pKa values of drug leads similar to CNBQ. However, UV-metric method can be conveniently and accurately utilized to determine the pKa values of drug leads of this class

6.0 CHAPTER-III

Title:
Development and validation of stability indicating assay test method to determine the content of aceclofenac in the pharmaceutical tablet dosage forms by RP-HPLC method.

6.1 Abstract

The purpose of this study was to develop and validate a versatile, rapid and precise stability indicating reversed phase HPLC test method to determine the content of aceclofenac (ACF) in the pharmaceutical tablet dosage form by RP-HPLC. ACF is decomposed under acidic and basic conditions, oxidative stress, in presence of light and also in different thermal stresses conditions. Chromatographic separation of ACF and its major and minor degradation products were successfully achieved on a C_{18} (250 mm length × 4.6 mm i.d., 5 μm particle size) column in an isocratic separation mode with mobile phase consisting of 0.07% of orthophosphoric acid and acetonitrile in the ratio of (68 : 32, v/v) at pH 7.0 ± 0.05, and the flow rate was maintained at 1.2 $mLmin^{-1}$ and the effluent was monitored at 275nm. Peak purity data of ACF was obtained using photodiode array detector (PDA) in the stressed sample chromatograms, which demonstrated the specificity of the method for the estimation of ACF in presence of degradation products. The method was validated with respect to linearity, precision, accuracy, selectivity, specificity, and ruggedness to assay ACF in tablets. The method was linear over the concentration ranges of 160–240 μgmL^{-1} (R^2 = 0.9993). Degradation products resulting from stress studies did not interfere with the detection of ACF and the assay method is thus stability indicating.

6.2 Introduction

ACF is chemically 2-{2-[2-(2,6-dichlorophenyl)aminophenyl]-acetyl}oxyacetic acid (Figure 49). It is white or almost white crystalline powder, practically insoluble in water, freely soluble in acetone and in dimethylformamide (DMF), soluble in ethyl alcohol and methanol. It has molecular formula of $C_{16}H_{13}Cl_2NO_4$ and molecular weight of 354.2 with a range of melting point 149-150°C. It is an NSAIDs that has been widely used for the treatment of arthritis.[1,2] It acts by blocking the action of cyclooxygenase, which is involved in the production of various chemicals in the body, some of which are known as prostaglandin.[3]

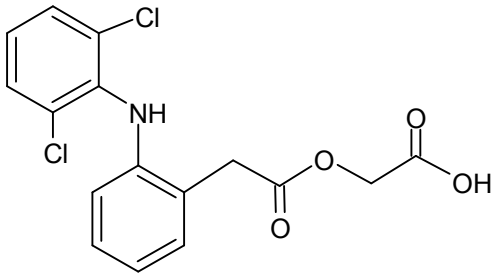


Figure 49: Chemical structure of ACF.

Forced degradation or stress testing of ACF was carried out according to stability test guidelines Q1A(R2) entitled "Stability Testing of New Drug Substances and Products" issued by ICH for establishing its inherent stability characteristics and for supporting the suitability of the proposed analytical procedure. The aim of stability testing is to prove how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors and is stated that the testing should include the effects of temperature, humidity where appropriate, oxidation, photolysis, and susceptibility to hydrolysis across a wide ranges of pH values. Regulatory agencies recommend the use of stability-indicating methods for the analysis of stability samples. Stability indicating method is a validated analytical procedure which is accurately and precisely measure the active ingredients without any co-elution of impurities, excipients and degradation products. Thus, stress studies

are required in order to generate the stressed samples, method development, and its validation.[4-7, 27]

Literature survey revealed that various methods have been reported for the estimation of ACF, such as striping voltammetric[8], titrimetric,[2,9] high performance thin layer chromatographic (HPTLC),[10,11] spectrophotometric,[12-18] spectrofluorimetric,[18] high-performance liquid chromatographic (HPLC)[1,3,19-25] and liquid chromatography–tandem mass spectrometric[26] methods for its determination in laboratory-prepared mixture, pharmaceutical preparations and biological matrices such as human plasma. As ACF is a non pharmacopoeial product, there are very few published methods that extensively focused on forced degradation study of ACF. Therefore, the aim of the present work is to develop a simple, precise, specific, accurate, cost-effective, and validated RP-HPLC method according to ICH guidelines Q2 (R1)[27] for the estimation and routine analysis of ACF in the presence of its degradants in pharmaceutical formulations.

6.3 Materials and Methods

6.3.1 Reagents and Materials.

ACF tablets were prepared in the Formulation Laboratory, Department of Pharmaceutical Technology, University of Dhaka, Bangladesh. Each film coated tablet contained aceclofenac (100 mg, based on 100% potency, APIChem, China) as an active ingredient, and microcrystalline cellulose/avicel PH 101 (Mingtai Chemical, Taiwan), croscarmellose sodium (Roswell Inc., USA), polyvinylpyrrolidone (BASF, Germany), colloidal silicon dioxide / aerosil-200 (Degussa, Germany), and magnesium stearate (Novochem GmbH, Germany) as excipients. HPLC grade acetonitrile was procured from Merck, India and reagent grade orthophosphoric acid (Sigma-Aldrich, Switzerland), hydrochloric acid (Merck, India), sodium hydroxide (Scharlu, Spain) and hydrogen peroxide (Scharlu, Spain) were used for analytical purposes. Milli-Q water was used to prepare the mobile phase.

6.3.2 HPLC Method

The chromatographic system used for the investigation was Shimadzu (Kyoto, Japan) prominence integrated with variable wavelength programmable photo diode array (PDA) detector composed of binary pump, degasser, auto injector, and column oven. The chromatographic analysis was performed in an isocratic separation mode on a C_{18}, 250 mm length × 4.6 mm i.d. with 5 μm particle size column. The mobile phase was a homogenous mixture of 0.07% v/v of orthophosphoric acid and acetonitrile in the ratio of (68 : 32, v/v) at pH 7.0 ± 0.05, pumped at a flow rate of 1.2 $mLmin^{-1}$. The column temperature was maintained at 30°C, and the detection wavelength was 275nm. Measurements were made with injection volume 10μL and the run time was 40 min for each injection of stressed sample. The retention time of ACF was about 7.0 min and the peak purity was obtained directly from the spectral analysis report.

Preparation of Mobile Phase:

Accurately measured 0.7mL of orthophosphoric acid was transferred and diluted with distilled water up to 1000mL. Then 680mL of this solution was mixed with 320mL of HPLC grade acetonitrile and mixed well. Finally the resulting solution was filtered using 0.2μm filter.

Preparation of Stock Solution of Standard Aceclofenac:

A stock solution of ACF was prepared at a concentration of 1.0$mgmL^{-1}$ in mobile phase. Accurately weighed 200mg of ACF was transferred to a 200mL volumetric flask and about 50mL of mobile phase was added. Then sonicated for 5 min for complete dissolution of drug. The solution was allowed to cool at room temperature and then the volume was made up to the mark with the same diluting solution.

Preparation of Assay Test Solution

For the analysis, not less than 20 tablets were taken and their average weight was calculated. The tablets were then crushed and powdered finely. To prepare assay sample solution, powdered sample equivalent to 100mg of ACF was weighed and

transferred to a clean and dry 100mL volumetric flask. About 25mL of mobile phase was added as diluting solution and shaken thoroughly to extract the drug from the excipients and then sonicated for 5 min for complete dissolution of drug. The solution was allowed to cool at room temperature and then the volume was made up to the mark with the same diluting solution. From this solution, 10mL was transferred to a clean and dry 50mL volumetric flask and the volume was made up to the mark with the same diluent. The solution was then filtered through Whatman filter paper (No. 42) and then finally filtered through 0.2 μm disk filter. The drug concentration of the resulting test solution was determined by comparing with standard solution. All determinations were conducted in triplicate.

6.4 Analytical Method Validation Report

To validated the test method, different analytical performance parameters such as system suitability, linearity, specificity, limit of detection, limit of quantitation, forced degradation study, accuracy, precision, solution stability and robustness were determined according to ICH issued analytical method validation guidelines Q2 (R1).[27]

6.4.1 System Suitability

To assess system suitability of the proposed method, repeatability, theoretical plates, tailing factor, and retention time of six replicate injections of standards were measured. Percentage relative standard deviation (%RSD), tailing factor and theoretical plate values were calculated in each case. The results (Mean ± %RSD of six replicates) of the chromatographic parameters in Table 18 indicating the good performance of the system.

Table 18: Chromatographic characteristics of system suitability solution.

Parameter	**Value (Mean ± %RSD)**
Peak area	3217711.2 ± 0.06
Tailing factor	1.109 ± 0.34
Theoretical plate	3132.419 ± 0.33
Retention time	6.9515 ± 0.03

6.4.2 Linearity

The linearity was evaluated by analyzing five working solutions of ACF over the concentration range 160 to 240µgmL^{-1} corresponding to 80 to 120% of nominal test concentration (200 µgmL^{-1}) for routine analysis of ACF. The linearity was evaluated by linear regression analysis, which was then evaluated by the least-square regression analysis. The representative linear equation was Y = 37370X - 493066 by plotting peak area (Y) versus the concentration (X) studied from 160 to 240 µgmL^{-1}. The correlation coefficient (R^2) value was 0.9993. Thus the method is considered to be linear.

6.4.3 Filter Paper Evaluation

To select the most suitable filter paper for filtration of the test solution the same test solution was filtered through different types of filter paper and compared with the result of the unfiltered (centrifuged) sample. The test solution was filtered through Whatman filter paper (No. 1, 41 and 42) and then finally filtered through 0.2 µm disk filter. From the study it is observed that Whatman filter paper (No. 42) is suitable for filtration of the test solution.

6.4.4 Specificity

The specificity of the developed method was determined by placebo analysis and checking peak purity of the test solution. From this study, it was observed that each component gave response separately with respect to retention time and passed peak

purity. No interference due to blank was observed in the chromatogram for blank solution (Figure 50). Good resolution was obtained between the drug and the degradation products formed under different stress conditions, also indicating good specificity of the method. The HPLC chromatograms recorded for the mixture of the inactive ingredients revealed no peaks within retention time around 6.95 min, and the peak purity was 99.99%, indicating ACF is clearly separated from the response of any interfering peak(s). Therefore, the method is considered to be specific.

6.4.5 Forced Degradation Study

Forced degradation study of SX were carried out using different medium (3% H_2O_2, 0.1 M HCl, and 0.1 NaOH, deionized water) for 1 h at 30°C, also the samples were treated with light for 5 days and heated (60°C and 105°C) for 1 h. RP-HPLC chromatograms and results obtained during forced degradation study are presented in the results and discussion section.

Sample preparation:

Two sample solutions were prepared, where 10mL of stock solution was diluted with 10mL of acid/base/10% H_2O_2 in two separated 50mL volumetric flask. These solutions were then subjected to heating at 30°C (±1°C) for 1 h. Samples were allowed to cool at room temperature and suitably diluted with diluting solution before analysis. At the same time, a blank sample was prepared in a 50mL volumetric flask by diluting 10mL of the respective medium with the diluting solution up to the mark. The samples were then filtered and subjected to HPLC analysis.

For photolytic degradation studies, two nominal standard solutions of ACF (200 $\mu g.mL^{-1}$) were prepared; and exposed to light in a photostability chamber for 5 days. For thermal degradation studies, active ACF was subjected to dry heat in petri-dishes at 60°C (±1°C), and 105°C (±2°C) for 1 h respectively. Then nominal solution of ACF (200 $\mu g.mL^{-1}$) were prepared by diluting with the mobile phase.

6.4.6 Precision

The repeatability (intra-day precision) of the method was assessed by performing replicate measurements (n=6) of ACF in the tablet dosage form and the intermediate precision (inter-day precision) of the method was assessed by performing replicate measurements (n=6) of ACF in the same lot of tablet dosage form by different analyst on different dates by different instrument. The results were expressed as %RSD of the measurements. %RSD of the repeatability were found 0.07% and %RSD of intermediate precision were found 0.25%. All the results were found within the acceptable limit of not more that 2.0%. Thus the method is considered to be precise.

6.4.7 Accuracy

Accuracy of the proposed method was studied by recovery experiments of the drug-matrix solution. This test was carried out by measuring five different drug solutions, three replicate of each, containing 160, 180, 200, 220, and 240 $\mu g.mL^{-1}$ of ACF standard solution added to ACF placebo solution, corresponding to 80, 90, 100, 110, and 120% of the nominal analytical concentration of ACF (200 $\mu g.mL^{-1}$), and the percent recoveries of ACF in drug-matrix form were calculated. All the results were found within the acceptance limit of 98.0% to 102%. The overall results of percent recoveries (mean ± %RSD of three replicates) of ACF drug-matrix solutions are plotted in Table 19, indicating good accuracy of the proposed method. The calculated recovery values of ACF ranged from 99.76% (±0.02%) to 101.03% (±0.02%) in drug-matrix solutions.

Table 19: Accuracy studies of ACF in drug-matrix solutions

Sample Name	% Recovery*(Mean ± %RSD)
80% Sample	99.7584±0.02
90% Sample	99.9490±0.04
100% Sample	100.0167±0.03
110% Sample	101.0299±0.02
120% Sample	100.6385±0.05
Mean	100.28
SD	0.53
%RSD	0.53

* Mean and %RSD value of three replicates.

6.4.8 Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. To determine the robustness of the current method, the pH of the buffer solution was assessed at 6.9 and 7.1 instead of 7.0. The effect of flow rate was studied at 1.1 and 1.3 $mL.min^{-1}$ instead of 1.2 $mLmin^{-1}$. The effect of column temperature was studied at 25° and 35°C instead of 30°C. The effect of mobile phase composition was assessed at (0.07% OPA : Acetonitrile = 66 : 34, v/v) and (0.07% OPA : Acetonitrile = 70 : 30, v/v) instead of (0.07% OPA : Acetonitrile = 68 : 32, v/v). The system suitability parameters under these conditions was calculated in all cases and found well within the acceptable limit (%RSD of peak area NMT 2.0%, Tailing factor NMT 2.0, and Theoretical plate NMT 2000).

6.4.9 Sample Solution Stability

To check the solution stability test sample solution was kept in room temperature and light and measured the peak area of the analyte after specific intervals of 3, 6, 12, 18 and 24 h and the percent difference between initial results were calculated. The test

sample solution were found to be stable up to 24 h at room temperature because the percent differences between initial results were 0.87% at 24 h.

6.5 Results and Discussion

Samples were analyzed by RP-HPLC equipped with photodiode array detector. The RP-HPLC method is found to be specific, precise, accurate and linear (R^2 = 0.9993) over a range of 80% to 120% of the target concentration level. Degradant peaks were well resolved from the main peak. The peak purity of the principal peak was greater than 0.99 in all stress conditions suggesting that there was no interference of degradants with the principal peak.

Figure 50-59 represents the HPLC Chromatograms obtained in forced degradation studies of ACF. From the blank chromatograms (Figure 50, 52, 54, 57) shows that placebo/blank has no interference on the ACF peak because there is no co-eluting peak at the retention time of ACF (~7.0 min) in the blank chromatograms. Treated sample chromatograms (Figure 51, 53, 55, 56, 58, 59) also show that there is no interference of degradants on ACF peak because all the degradants elute separately and peak purity of the ACF peak proves that there is no co-eluting peak with ACF peak.

The drug was very susceptible to decompose under hydrolytic stress. 22.6% drug is degraded in acidic media and 70.8% in alkaline media. It was also shown liability in oxidative stress (degradation: 25.1%) and exposure to light (degradation: 20.7%). The drug was found stable under dry heat at 80°C (degradation: 0.2%) but unstable at 105°C (degradation: 60.7%). In all cases, chromatographic peak purity data of ACF were obtained from the spectral analysis report. The peak purity value was found to be greater than 99.99%, indicating a homogenous peak of ACF and confirming the absence of other substance in the same retention time (RT).

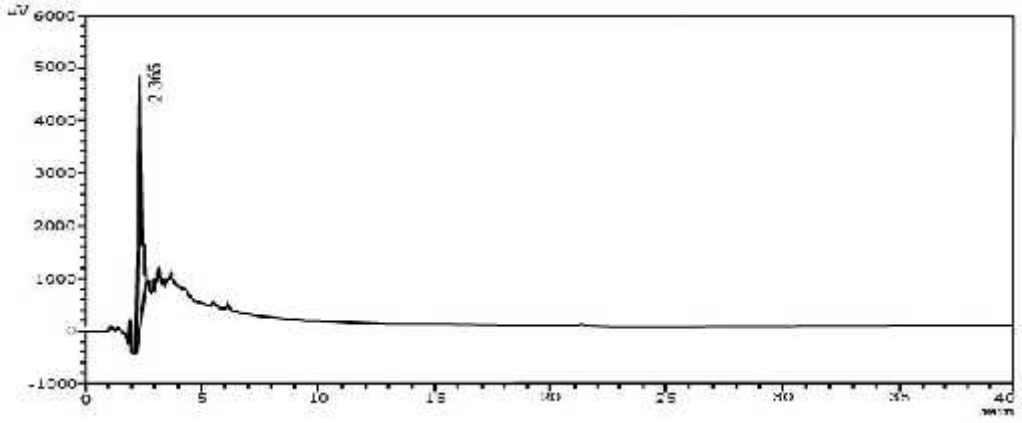


Figure 50: Chromatogram of 0.1 N HCl stressed blank sample.

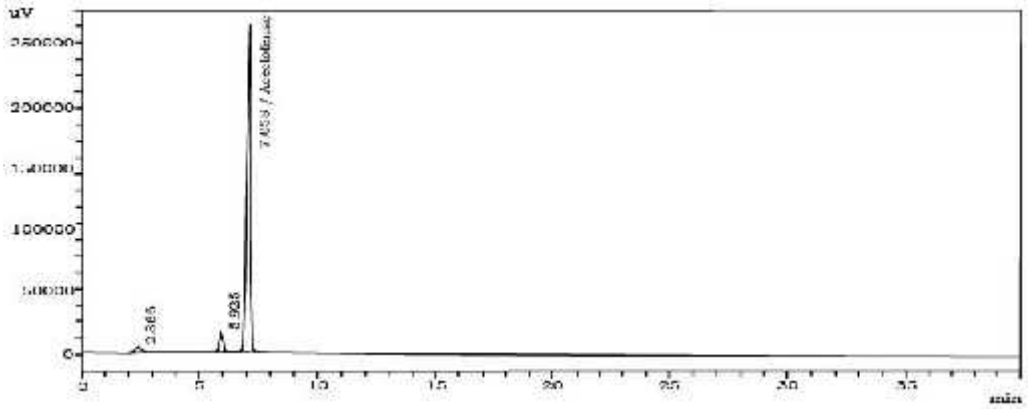


Figure 51: Chromatogram of 0.1 N HCl stressed ACF solution at 30°C (±1°C) for 1 h.

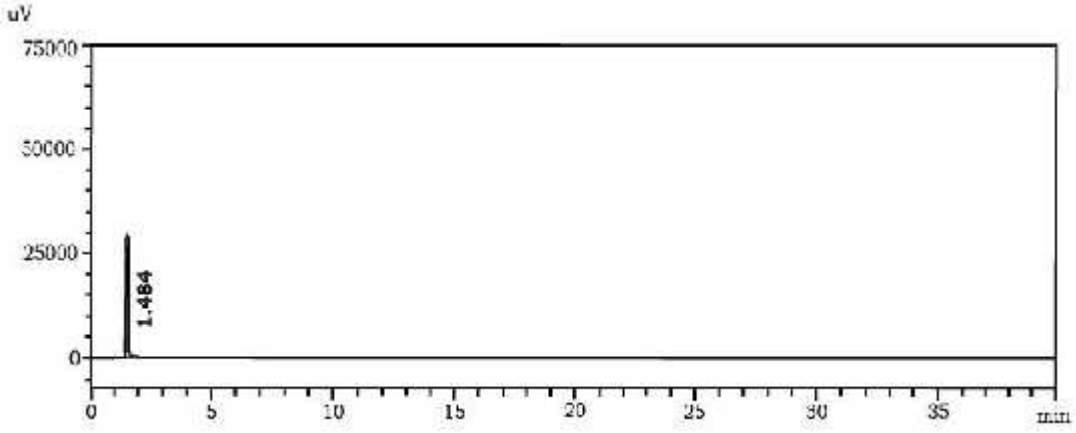


Figure 52: Chromatogram of 0.1 N NaOH stressed blank sample.

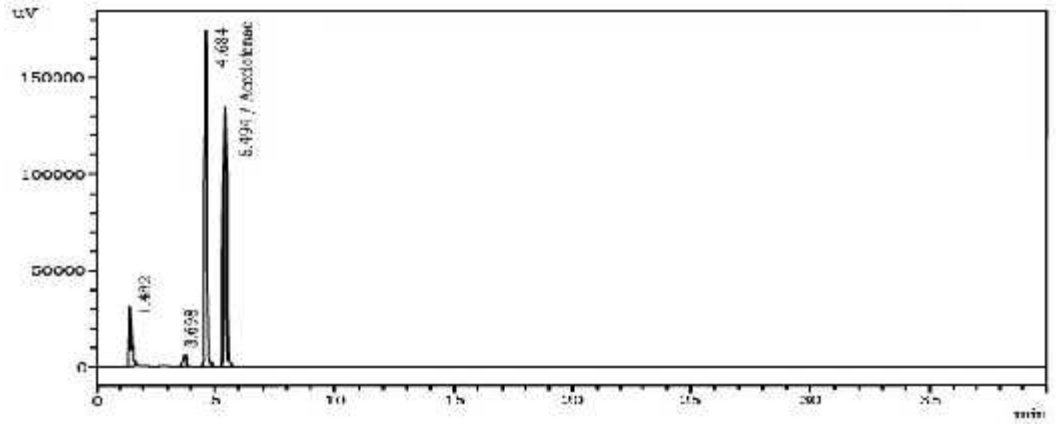


Figure 53: Chromatogram of 0.1 N NaOH stressed ACF solution at 30°C (±1°C) for 1 h.

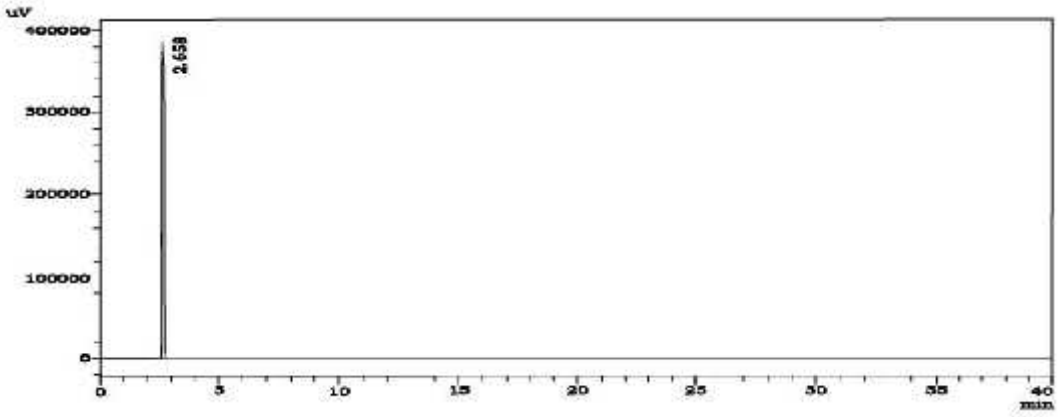


Figure 54: Chromatogram of 10% H_2O_2 stressed blank sample.

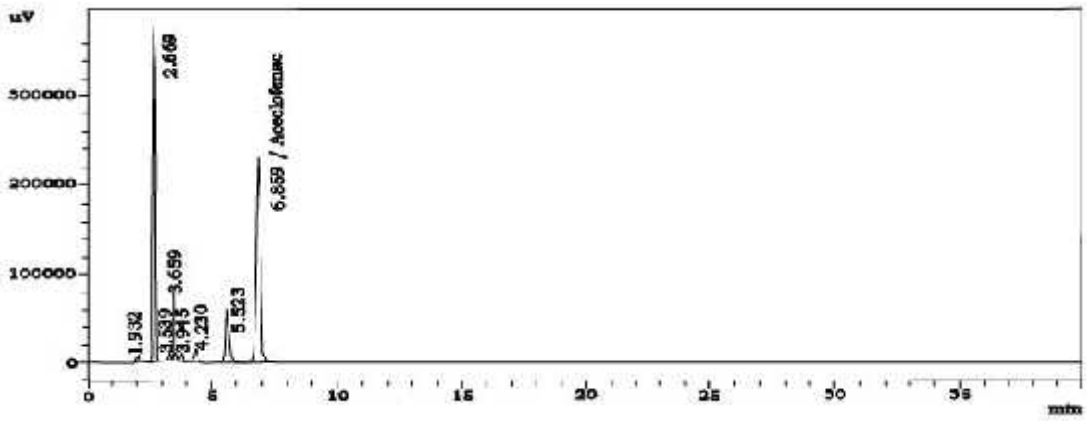


Figure 55: Chromatogram of 10% H_2O_2 stressed ACF solution at 30°C (±1°C) for 1 h.

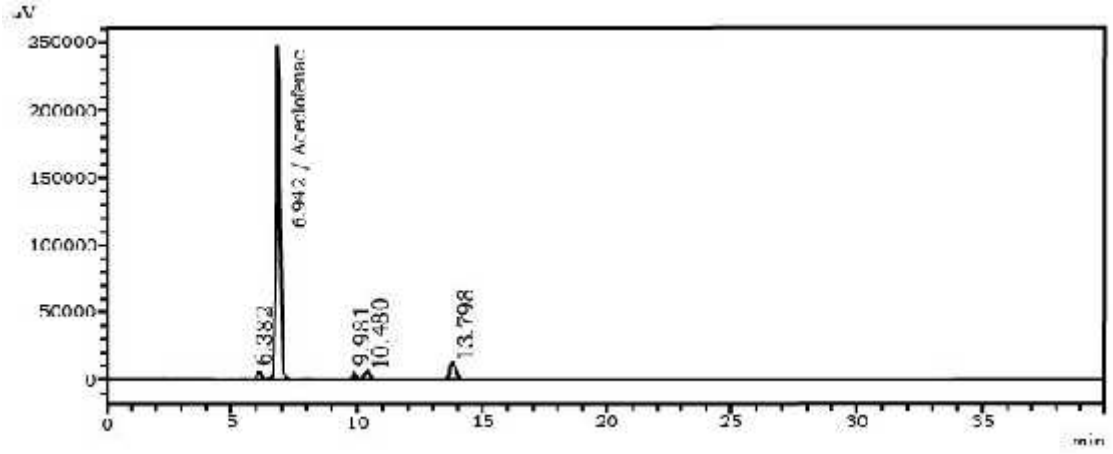


Figure 56: Chromatogram of ACF solution in presence of light for 5 days.

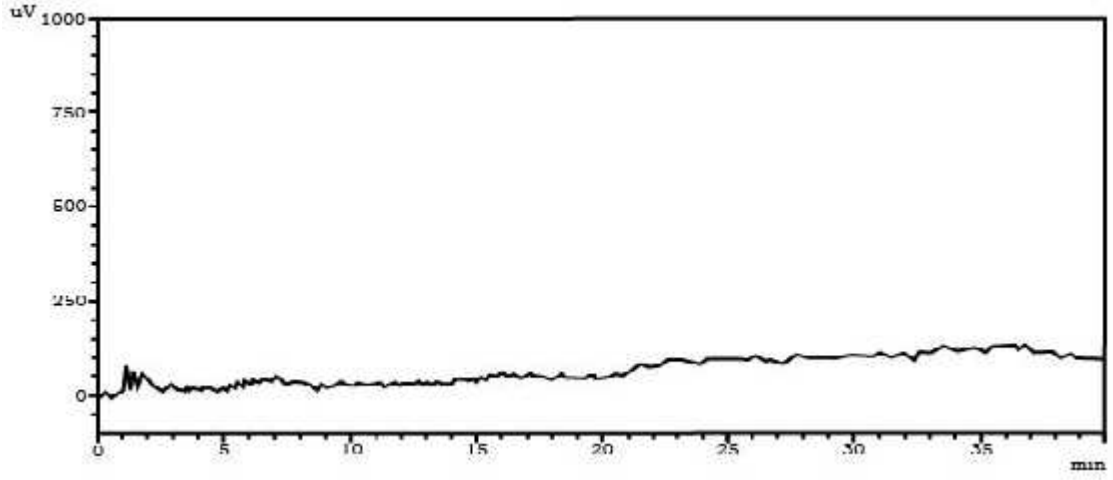


Figure 57: Chromatogram of thermal stressed blank sample

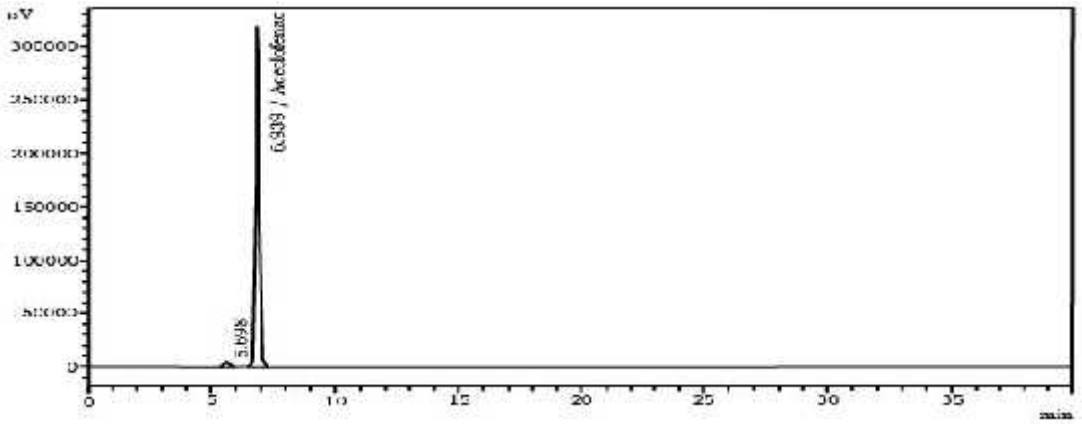


Figure 58: Chromatogram of thermal stressed ACF at 60°C (±1°C) for 1 h.

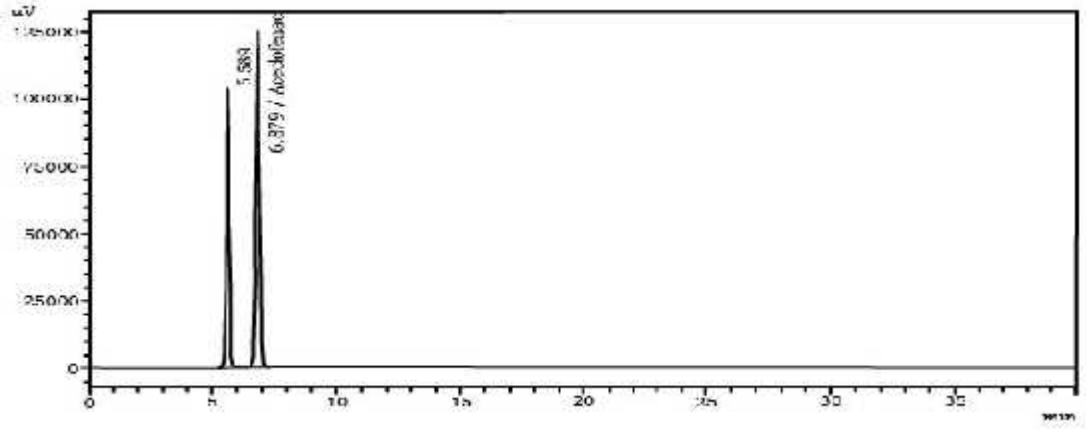


Figure 59: Chromatogram of thermal stressed ACF at 105°C (±2°C) for 1 h.

6.6 Conclusion

A stability indicating method developed to determine the content of aceclofenac in the pharmaceutical tablet dosage form is specific, precise, linear, accurate, and rugged enough. The method adopted test sample solution is found to be stable up to 24 h at room temperature. Hence, this method can be considered valid for its intended purpose to establish the quantity of ACF with consistent and reproducible results during routine analysis in quality control and stability studies.

7.0 CHAPTER-IV

Title:

Development and validation of the stability indicating test method to determine the content of salmeterol xinafoate and its organic impurities in pharmaceutical pressurized inhalation dosage form by RP-HPLC.

7.1 Abstract

The purpose of this study was to develop and validate a stability indicating RP-HPLC test method to determine the content of drug substance and its organic impurities of salmeterol xinafoate (SX) in inhaler dosage form. Chromatographic separation of SX and its known impurities were successfully achieved on a stainless still column packed with C18 column (250 mm × 4.6 mm, 5 µm) in a gradient separation mode with mobile phase consisting of phosphate buffer (0.015 M KH_2PO_4 at pH 6.8 ± 0.05) and methanol. Flow rate was maintained at 1.0 $mL.min^{-1}$ and the effluent was monitored at 278nm. As per ICH guidelines, the method was validated with respect to suitability, specificity, linearity, precision, accuracy, and robustness. Specificity of the method in presence of impurities and degradation products was evaluated by peak purity testing using photodiode array (PDA) detector. In order to check the peak purity, samples were kept under different stressed conditions (acidic, basic, oxidative, thermal and light) and were spiked with known impurities. The method was found to be linear (R^2<0.99) over the concentration ranges of LOQ to 120% of specification level. The RP-HPLC method was also found to be accurate and precise with percentage recovery values between 98.0% to 102.0% and 90.0% to 110.0%, and percentage RSD values less than 2.0% and 10.0% for SX and its all impurities, respectively. Test sample solution is found to be stable up to 24 h at room temperature. Hence, this method can be used to quantify the amount of SX and its impurities in the drug product during product development and routine analysis.

7.2 Introduction

Salmeterol xinafoate is chemically known as (1*RS*)-1-[4-Hydroxy-3-(Hydroxymethyl) phenyl]-2-[[6-(4-phenylbutoxy) hexyl] amino] ethanol 1-hydroxynaphthalene-2-carboxylate (Figure 60). Salmeterol Xinafoate is white or almost a white powder. It is practically insoluble in water, soluble in methanol, and slightly soluble in anhydrous ethanol. It has a molecular formula of $C_{36}H_{45}NO_7$ and a molecular weight of 604.[1]

SX is a β_2-adrenergic receptor agonist, extensively used as a bronchodilator for the management of asthma or chronic obstructive pulmonary diseases (COPD). It is not suitable for acute asthma attack treatment because of its long onset of action. Its long duration of action is because of its lipophilicity and this property adds to its presence at the action site proving SX to be more reliable as compared to other β2 adrenoceptor agonist drugs.[2,3,4,5] The working of SX involves opening up the air passages in lungs allowing the air to freely flow into the lungs thus alleviating the symptoms such as coughing, wheezing and breathlessness.[2]

The presence of impurities consisting of the active pharmaceutical ingredients (APIs), such as unreacted starting materials, reagents, byproducts and degraded products, causing adverse effects on the human health because of their mutagenic or carcinogenic effects. Thus, quantification and qualification of impurities in drug substances and drug products is of significant essence. Therefore, ICH guidelines has to be followed during drug development and production.[6] As per the ICH guidelines, if the impurities presence exceeds identification thresholds in the drug product, it must be identified and if exceeds qualification thresholds in the drug product, it must be qualified and characterized.[7] Seven known impurities present in salmeterol have been reported in British Pharmacopoeia monograph.[1] The structures of salmeterol along with its known impurities are shown in figure 60-68.

Quality of drug product is invariably and uncontrollably influenced by time along with various environmental factors such as temperature, humidity and drug excipients interaction. As a result, in course of time, degradation impurities may be generated in the drug product causing it to produce health effects on patients. The use of stability

indicating methods is therefore, highly recommended by the drug regulatory bodies for analysis of the samples to determine the probable amount of impurities that will be generated during the drug's shelf-life. Literature survey showed that there are few stability indicating methods reported for the estimation of salmeterol and for the determination of impurities from the SX and its inhaler powder dosage form.[8,9,10] However, no method has been found for the estimation of salmeterol and its known impurities from the its pressurized inhalation dosage form. Therefore, the aim of the present work is to develop and validate an RP-HPLC assay and impurities test method for determining the amount of salmeterol and its known impurities from the salmeterol pressurized inhalation dosage form .

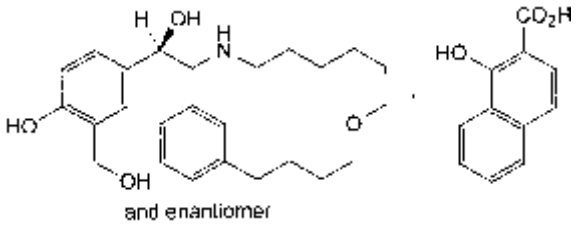


Figure 60: Chemical structure of salmeterol xinafoate.

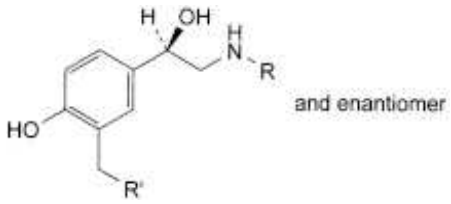


Figure 61: Chemical structure of salmeterol impurity A. (R = $[CH_2]_4$-C_6H_5, R´ = OH)

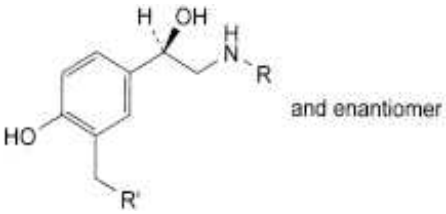


Figure 62: Chemical structure of salmeterol impurity B. (R = $[CH_2]_6$-*O*-$[CH_2]_2$-C_6H_5, R´ = OH)

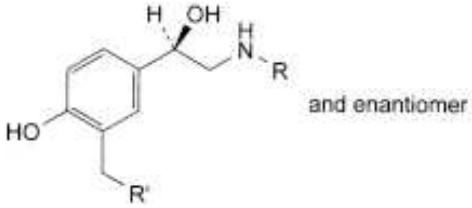


Figure 63: Chemical structure of salmeterol impurity C. (R = $[CH_2]_6$-*O*-$[CH_2]_3$-C_6H_5, R′ = OH)

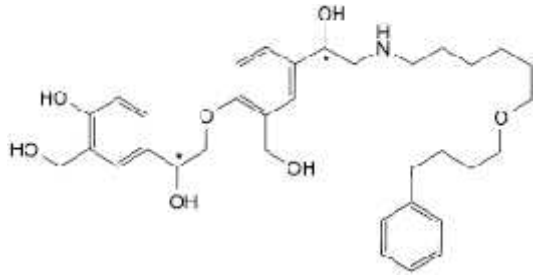


Figure 64: Chemical structure of salmeterol impurity D

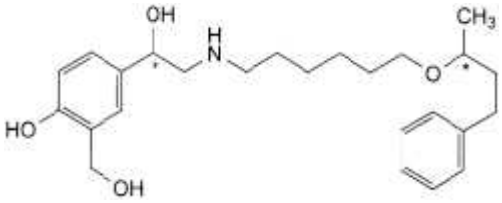


Figure 65: Chemical structure of salmeterol impurity E.

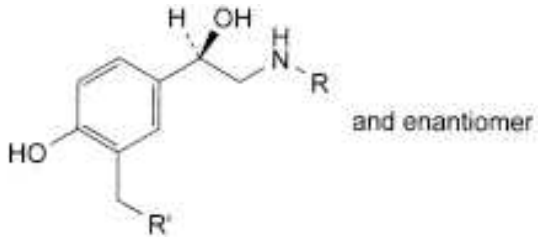


Figure 66: Chemical structure of salmeterol impurity F (R = $[CH_2]_6$-*O*-$[CH_2]_4$-C_6H_5, R′= H)

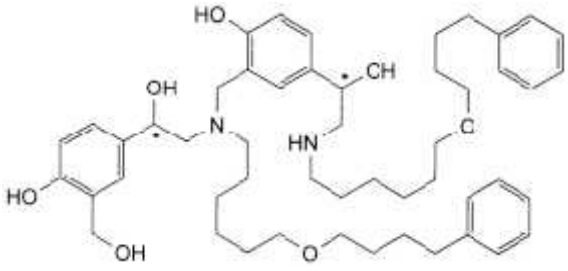


Figure 67: Chemical structure of salmeterol impurity G.

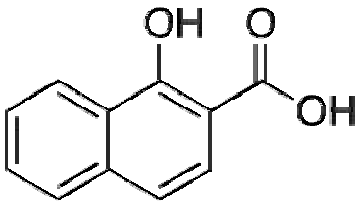


Figure 68: Chemical structure of Xinafoic acid.

7.3 Materials and Method

7.3.1 Reagents and Materials.

HPLC grade methanol (Merck, India) and reagent grade potassium dihydrogen phosphate (Merck, India), orthophosphoric acid (Sigma-Aldrich, Switzerland), hydrochloric acid (Merck, India), sodium hydroxide (Scharlu, Spain) and hydrogen peroxide (Scharlu, Spain) were used for analytical purposes. Milli-Q water was used to prepare the mobile phase. The following materials: Salmeterol Xinafoate, Xinafoic acid, Salmeterol Impurity A, Salmeterol Impurity B, Salmeterol Impurity C, Salmeterol Impurity E, Salmeterol Impurity D, Salmeterol Impurity F, Salmeterol Impurity G, placebo inhalers, and Salmeterol Inhalers were received as gift samples from Beximco Pharmaceuticals Limited, Bangladesh. Each single dosage form contained Salmeterol Xinafoate (25µg, based) as an active ingredient Oleic Acid, Dehydrated Alcohol and 1,1,1,2 Tetrafluoroethane (HFA 134a) as excipients.

7.3.2 Method

The chromatographic system used for the investigation was Shimadzu (Kyoto, Japan) Prominence integrated with variable wavelength programmable photo diode array (PDA) detector composed of binary pump, degasser, auto injector, and column oven. The Chromatographic separation of SX and its known impurities were successfully achieved on a stainless still column packed with C18 column (250 mm × 4.6 mm, 5 µm) in a gradient separation mode with mobile phase consisting of phosphate buffer (0.015 M KH_2PO_4 at pH 6.8 ± 0.05) and methanol (Table 20). Flow rate was maintained at 1.0mL.min^{-1} and the effluent was monitored at 278nm. Measurements

were made with injection volume of 100µL and the run time was 120min for each injection. The peak purity was obtained directly from the spectral analysis report.

Table 20: Gradient flow of mobile phase

Time (min)	Buffer Solution	Methanol
0	90	10
20	50	50
45	40	60
60	30	70
80	10	90
90	10	90
120	90	10

7.3.2.1 Preparation of Buffer Solution

Accurately measured and diluted 2.04 g of potassium dihydrogen phosphate up to 1000 mL with distilled water. Finally, the pH was adjusted at 6.8 ±0.05 with 1.0 M sodium hydroxide solution and the resulting solution was filtered using 0.45µm GHP filter.

7.3.2.2 Preparation of Diluent

Buffer Solution: Methanol (50:50)

7.3.2.3 Preparation of System Suitability Solution:

0.1125 mg/mL of Salmeterol Xinafoate and 0.001125 mg/mL of Salmeterol Impurity E in Diluent.

7.3.2.4 System Suitability Specifications:

a) The resolution between the peaks due to impurity E and salmeterol is more than 1.5
b) The tailing factor for salmeterol peak is not more than 2.0
c) The plate count for salmeterol peak should be not less than 2000.

7.3.2.5 Preparation of Placebo Solution

The canister was shaken and 90 deliveries were discharged from the placebo can into a dry 50ml beaker. To this solution was added 10 ml of methanol and 10 ml of buffer solution under sonication.

7.3.2.6 Preparation of Test Solution

The canister was shaken and 90 deliveries were discharged from the inhaler can into a dry 50ml beaker. To this solution was added 10 ml of methanol and 10 ml of buffer solution under sonication.

7.3.2.7 Calculation

Disregard any peak area equal to or less than 0.05% of the area of salmeterol peak in the chromatogram obtained with test solution. Disregard the peak due to xinafoic acid and any peaks due to the placebo solution.

The % of each impurity is obtained directly from the chromatogram.

$$\%\ \text{of known/unknown impurity} \frac{A_{u/k}}{A_T} \times \frac{100}{RRF}$$

Where,

Au/k = Area of known/unknown Impurity peak in the chromatogram obtained with test sample.

AT = Sum of the area of all known/unknown impurities peaks and salmeterol peak in the chromatogram obtained with test sample.

RRF = Relative Response Factor calculated from the linearity data (RRF=slope of the impurity/Slope of the API)

Table 21: Relative Retention Time and Relative Response Factor

Sample Name	**Relative Retention Time**	**Relative Response Factor**
Xinafoic acid	0.32	--
Salmeterol Impurity A	0.37	--
Salmeterol Impurity B	0.60	--
Salmeterol Impurity C	0.81	--
Salmeterol Impurity E	0.96	--
Salmeterol	1.00	1.00
Salmeterol Impurity D	1.06	--
Salmeterol Impurity F	1.18	--
Salmeterol Impurity G	1.39	0.77

7.4 Analytical Method Validation Report

To validated the test method, different analytical performance parameters such as system suitability, linearity, specificity, limit of detection, limit of quantitation, forced degradation study, accuracy, precision, solution stability and robustness were determined according to ICH issued analytical method validation guidelines Q2 (R1).[10]

7.4.1 System Suitability

To assess system suitability of the proposed method, theoretical plates, tailing factor, and resolution between impurity E and salmeterol of system suitability solution was injected and measured. Percentage relative standard deviation (%RSD), tailing factor and theoretical plate values were calculated in each case. The results of the chromatographic parameters in Table 22 indicating the good performance of the system. HPLC Chromatogram for system suitability solution is shown in figure 69.

Table 22: Chromatographic characteristics of system suitability solution.

Parameters	Results
Resolution Between Impurity E and Salmeterol	1.85
Tailing Factor	1.7
Theoretical Plate	38200
%RSD of area	1.23%

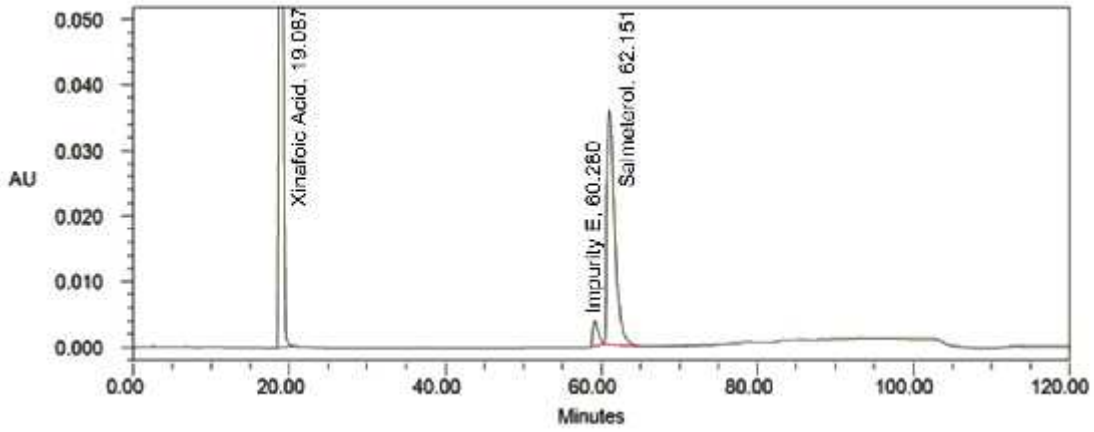


Figure 69. HPLC Chromatogram for system suitability solution

7.4.2 Filter paper Evaluation

To select the most suitable filter for filtration of the test solution the same test solution was filtered through different types of filter and compared with the result of the unfiltered (centrifuged) sample. The test solution was filtered through 0.45 µm disk filter GHP and PTFE disk filter. From the study it is observed that 0.45 µm disk filter (GHP) is suitable for filtration of the test solution.

7.4.3 Specificity

The specificity of the developed method was determined by placebo analysis and checking peak purity of the unspiked (Figure 71) and spiked (Figure 72) test solutions. Spiked test solution was prepared by adding all the known impurities with the test solution. Retention time for all known impurities were confirmed by injecting all

impurities separately. From this study, it is observed that each component gave response separately with respect to retention time and passed peak purity. No interference due to blank was observed in the chromatogram for blank solution (Figure 70). Good resolution was obtained between the drug and the degradation products formed under different stress conditions, also indicating good specificity of the method. The HPLC chromatograms recorded for the mixture of the inactive ingredients revealed no peaks within retention time each compound, and the peak purity was 99.99%, indicating all compounds is clearly separated from the response of any interfering peak(s). Therefore, the method is considered to be specific.

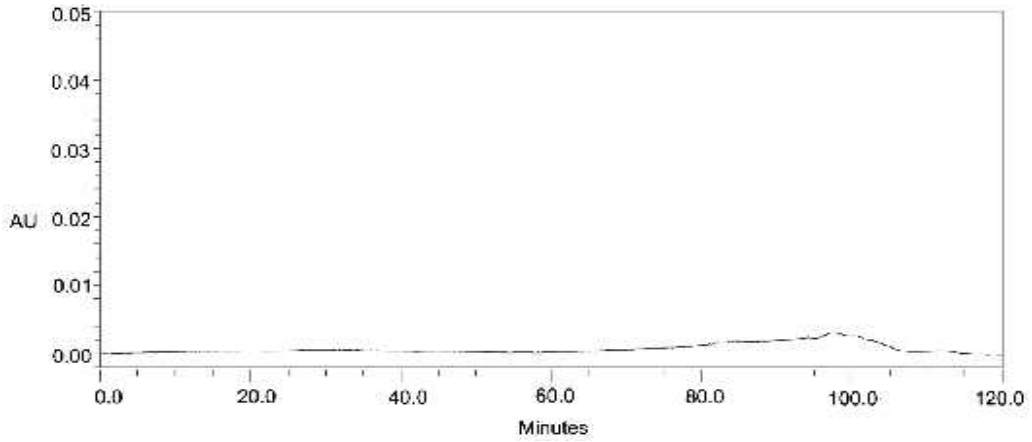


Figure 70. HPLC Chromatogram for the blank solution

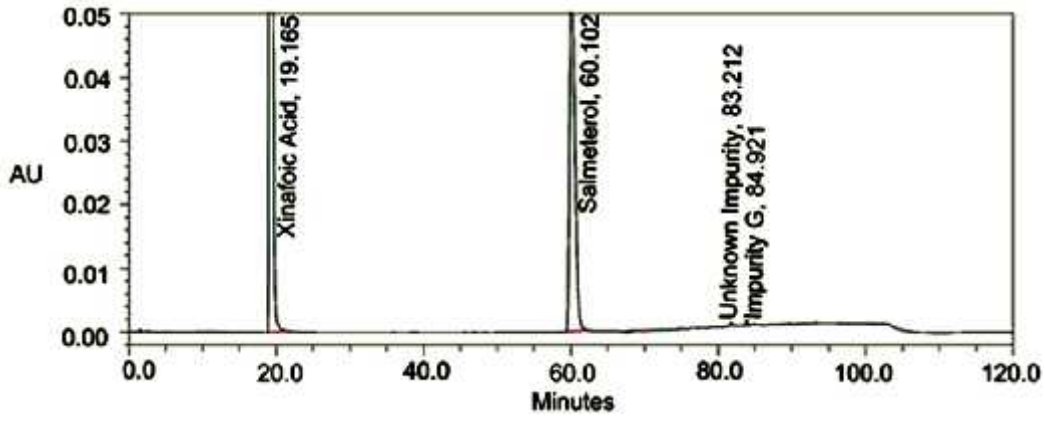


Figure 71. HPLC Chromatogram for the unspiked test solution

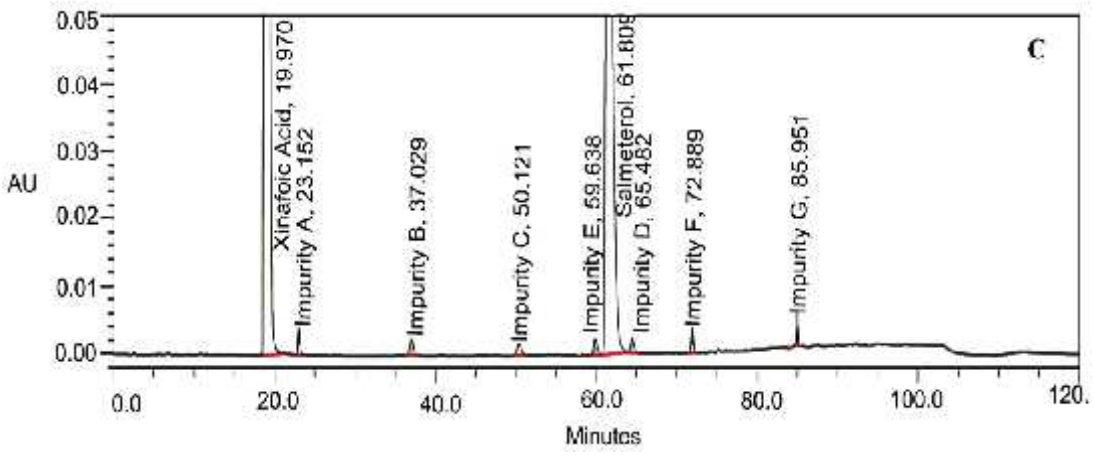


Figure 72. HPLC Chromatogram for the spiked test solution

7.4.4 Linearity

The linearity was evaluated by analyzing different concentration of SX and its all impurities over the concentration range of LOQ to 200% of nominal test concentration of 0.1125mg/mL of salmeterol and 0.001125mg/mL of salmeterol impurities. The linearity was evaluated by linear regression analysis, which was then evaluated by the least-square regression analysis. The representative linear equations shows in table 23 which was obtained by plotting peak area (Y) versus the concentration (X) studied. The correlation coefficient (R^2) value were 0.99. Thus the method is considered to be linear.

Table 23: Linear equation for all impurities

Sample Name	Regression Equation	Correlation coefficient (R^2)
Salmeterol	y = 20511x - 5060	R^2 = 0.9999
Salmeterol Impurity A	y = 19763x - 9	R^2 = 0.9997
Salmeterol Impurity B	y = 21326x - 1769	R^2 = 0.9993
Salmeterol Impurity C	y = 22693x - 6271	R^2 = 0.9969
Salmeterol Impurity E	y = 22041x + 436	R^2 = 0.9999
Salmeterol Impurity D	y = 21211x - 4632	R^2 = 0.9993
Salmeterol Impurity F	y = 21953x - 493	R^2 = 0.9996
Salmeterol Impurity G	y = 15739x - 6489	R^2 = 0.9936

7.4.5 Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated in accordance with the 3.3s/m and 10s/m criteria, respectively, according to ICH Q2 (R1) recommendations, where 's' is the standard deviation of the peak area and 'm' is the slope of the calibration curve, determined from linearity investigation. Figure 73 and 74 represents the HPLC Chromatograms obtained from calculated limit of detection level, and B: limit of quantitation level (Table 24).

Table 24: Limit of detection and limit of quantitation

Sample Name	LOD (%w/w)	LOQ (%w/w)
Salmeterol Impurity A	0.05	0.15
Salmeterol Impurity B	0.15	0.45
Salmeterol Impurity C	0.25	0.75
Salmeterol Impurity E	0.20	0.60
Salmeterol	0.11	0.33
Salmeterol Impurity D	0.18	0.54
Salmeterol Impurity F	0.05	0.15
Salmeterol Impurity G	0.20	0.60

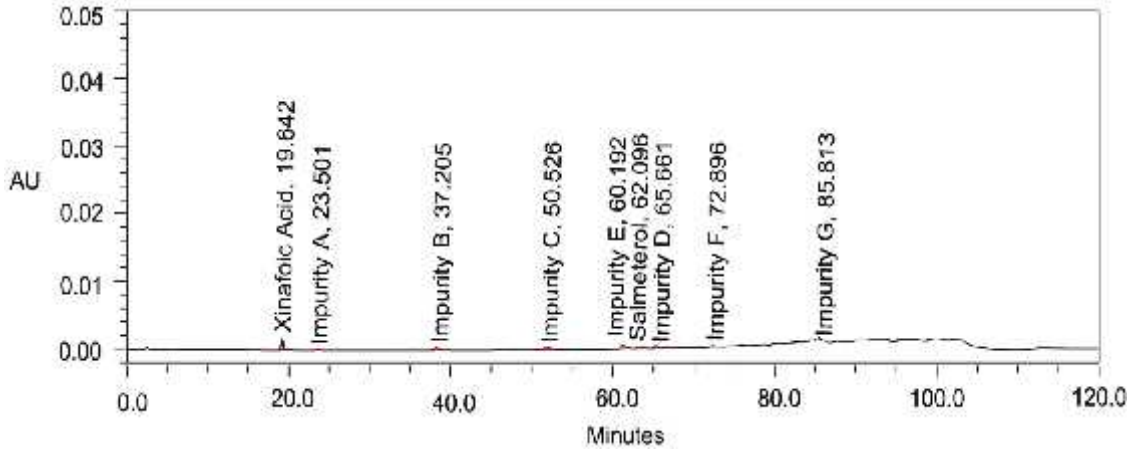


Figure 73. HPLC Chromatogram for limit of detection level

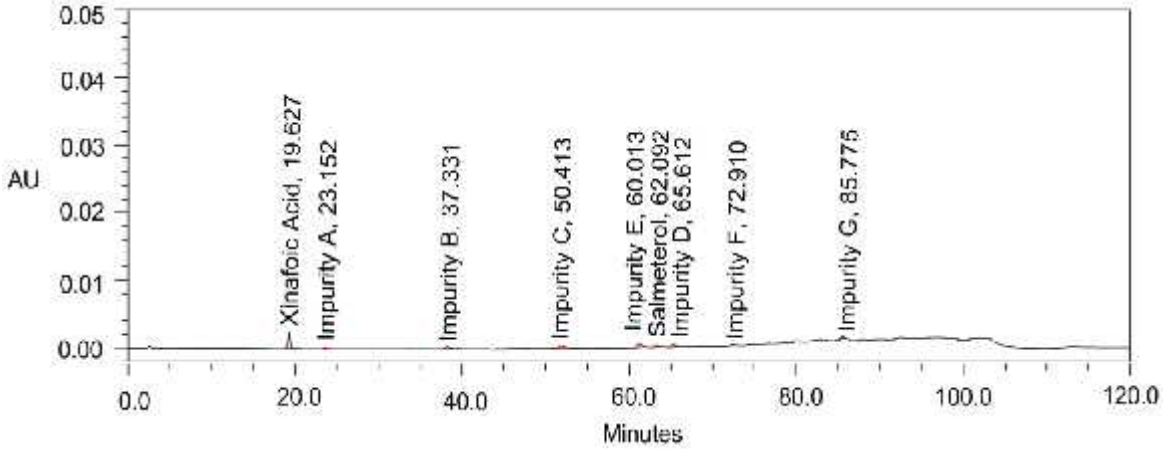


Figure 74. HPLC Chromatogram for limit of quantitation level

7.4.6 Forced Degradation Study

Forced degradation study of SX were carried out using different medium (3% H_2O_2, 0.1 M HCl, and 0.1 NaOH) by refluxing the solution on boiling water bath for 15 Minutes, also the samples were treated with light for 5 days and heated (105°C) for 1 hr. RP-HPLC chromatograms and results obtained during forced degradation study are presented in the results and discussion section.

Stock Solution

0.45mg/mL of salmeterol stock solution was prepared by dissolving in methanol with proper mixing.

Sample preparation:

Two sample solutions were prepared, where 5mL of stock solution was diluted with 5mL of acid/base/3% H_2O_2 in two separated 20mL volumetric flask. These solutions were then subjected to reflux on boiling water bath for 15 Minutes. Samples were allowed to cool at room temperature and suitably diluted with diluting solution before analysis. At the same time, a blank sample was prepared in a 20mL volumetric flask by diluting 5mL of the respective medium with the diluting solution up to the mark. The samples were then filtered and subjected to HPLC analysis.

For photolytic degradation studies, nominal standard solutions of SX (0.1125mg/mL) were prepared; and was exposed to light in a photostability chamber for 5 days. For thermal degradation studies, active SX was subjected to dry heat in petri-dishes at 105°C (±2°C) for 1 hr. Then nominal solution of SX (0.1125 mg/mL) was prepared by diluting with the diluent. Percentage degradation and peak purity of the SX obtained from forced degradation studies were presented in table 25.

Table 25: %Degradation and peak purity of the SX

Medium	% Degradation	Peak purity
Acid	>41.5%	>0.999
Base	>8.3%	>0.999
Oxidation	>6.9%	>0.999
Light	>0.5%	>0.999
Heat	>1.9%	>0.999

7.4.7 Precision

The repeatability (intra-day precision) of the method was assessed by performing replicate measurements (n=6) of the test sample spiked with all known impurities. The intermediate precision (inter-day precision) of the method was assessed by performing

replicate measurements (n=6) of test sample spiked with all known impurities by different analyst on different dates by different instrument. The results were expressed as %RSD of the measurements. All the results were found within the acceptance limit of less than 2.0% and 10.0% for SX and its all impurities. Thus the method is considered to be precise.

Table 26: Precision studies of SX and its all impurities

Sample Name	Precision (%RSD)	Intermediate precision (%RSD)
Salmeterol	0.28%	0.19%
Salmeterol Impurity A	2.25%	0.47%
Salmeterol Impurity B	3.18%	1.27%
Salmeterol Impurity C	4.16%	2.56%
Salmeterol Impurity E	1.98%	1.39%
Salmeterol Impurity D	2.56%	2.78%
Salmeterol Impurity F	1.69%	0.97%
Salmeterol Impurity G	1.49%	3.25%

7.4.8 Accuracy

Accuracy of the proposed method was studied by recovery experiments of the drug-matrix solution. This test was carried out by measuring three different drug solutions, three replicate of each, containing 50, 100, and 200% of the nominal analytical concentration and the percent recoveries of SX and its all impurities were calculated. All the results were found within the acceptance limit of 98.0% to 102.0% and 90.0% to 110.0%, for SX and its all impurities, respectively. The overall results of percent recoveries (mean ± %RSD of three replicates) are plotted in Table 27, indicating good accuracy of the proposed method.

Table 27: Accuracy studies of SX and its all impurities

Sample Name	%Recovery (Mean ± %RSD)
Salmeterol	98.9 ± 0.89
Salmeterol Impurity A	96.8 ± 3.12
Salmeterol Impurity B	98.6 ± 2.98
Salmeterol Impurity C	103.9 ± 1.87
Salmeterol Impurity E	101.3 ± 2.09
Salmeterol Impurity D	92.5 ± 3.01
Salmeterol Impurity F	96.1 ± 1.97
Salmeterol Impurity G	98.8 ± 1.68

7.4.9 Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. To determine the robustness of the current method, the pH of the buffer solution was assessed at 6.7 and 7.0 instead of 6.8. The effect of flow rate was studied at 0.9 and 1.1mLmin^{-1} instead of 1.0mL.min^{-1}. The effect of column temperature was studied at 35° and 45°C instead of 40°C. The system suitability parameters under these conditions were calculated in all cases and found well within the acceptable limit (Resolution between Impurity E and Salmeterol NLT 1.5, Tailing factor NMT 2.0, and Theoretical plate NMT 2000).

7.4.10 Sample Solution Stability

To check the solution stability test sample solution was kept in room temperature and light, and measured the peak area of the analyte after specific intervals of 3, 6, 12, 18 and 24hrs and the percent difference between initial results were calculated. The test sample solution were found to be stable up to 24hr at room temperature because the percent differences between initial results were within the limit of not more than 2.0% and 10.0% SX and its all impurities at 24 hours, respectively (Table 28).

Table 28: Sample Solution Stability studies of SX and its all impurities

Sample Name	Solution Stability (%Degradation at 24hr)
Salmeterol	1.01%
Salmeterol Impurity A	0.90%
Salmeterol Impurity B	0.87%
Salmeterol Impurity C	4.92%
Salmeterol Impurity E	6.18%
Salmeterol Impurity D	2.76%
Salmeterol Impurity F	1.67%
Salmeterol Impurity G	3.9%

7.5 Results and Discussion

Samples were analyzed by RP-HPLC equipped with photodiode array detector. The RP-HPLC method is found to be specific, precise, accurate and linear ($R^2 > 0.99$) over a range of LOQ to 200% of the target concentration level for SX and its known impurities. Degradation peaks were well resolved from the main peak. The peak purity of the principal peak was greater than 0.99 in all stress conditions suggesting that there was no interference of degradants with the principal peak.

Figure 75-83 represents the HPLC chromatograms obtained in forced degradation studies of ACF. From the blank chromatograms (Figure 75, 77, 79, 81) shows that placebo/blank has no interference on the SX and its known impurities peak because there is no co-eluting peak at the retention time of SX and its known impurities peak in the blank chromatograms. Treated sample chromatograms (Figure 76, 78, 80, 82, 83) also shows that there is no interference of degradants on SX peak because all the degradants elute separately and peak purity of the SX peak proves that there is no co-eluting peak with SX peak.

The drug was very susceptible to decompose under acidic hydrolytic stress. 41.5% drug is degraded in acidic media. It was also shown liability in alkaline hydrolytic stress (degradation: 8.3%) and oxidative stress (degradation: 6.9%). The drug was

found to be stable under dry heat (degradation: 1.9%) and exposure to light (degradation: 0.5%). In all cases, chromatographic peak purity data of SX were obtained from the spectral analysis report. The peak purity value was found to be greater than 99.99%, indicating a homogenous peak of SX and confirming the absence of other substance in the same retention time (RT).

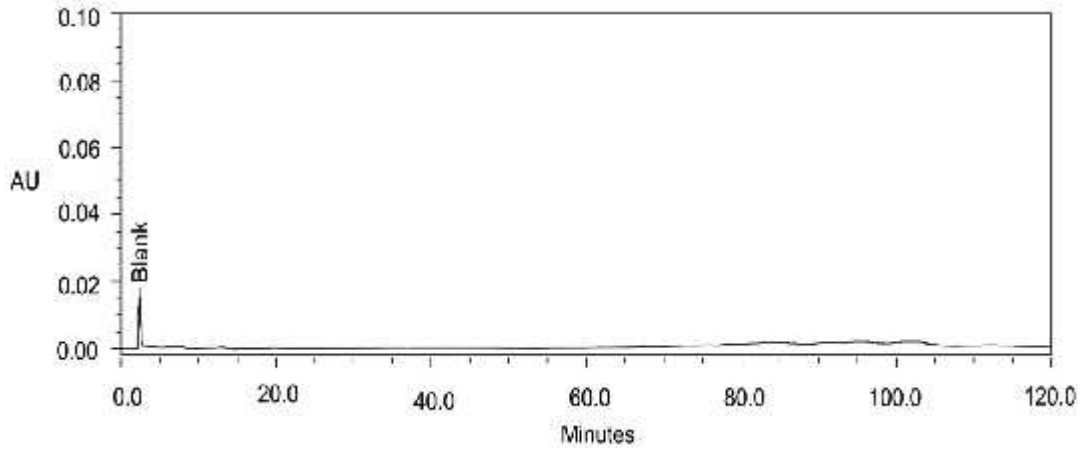


Figure 75: Chromatogram of 0.1 N HCl stressed blank sample.

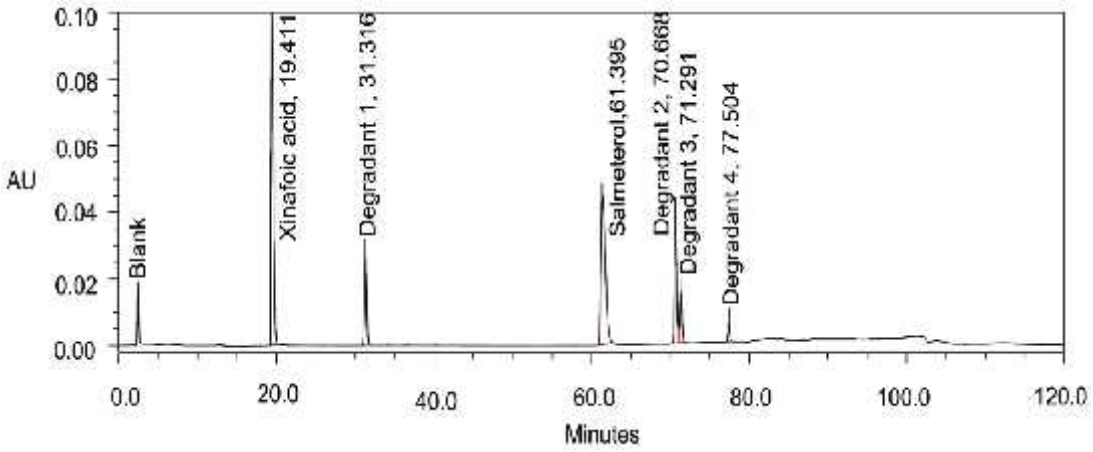


Figure 76: Chromatogram of 0.1 N HCl stressed solution

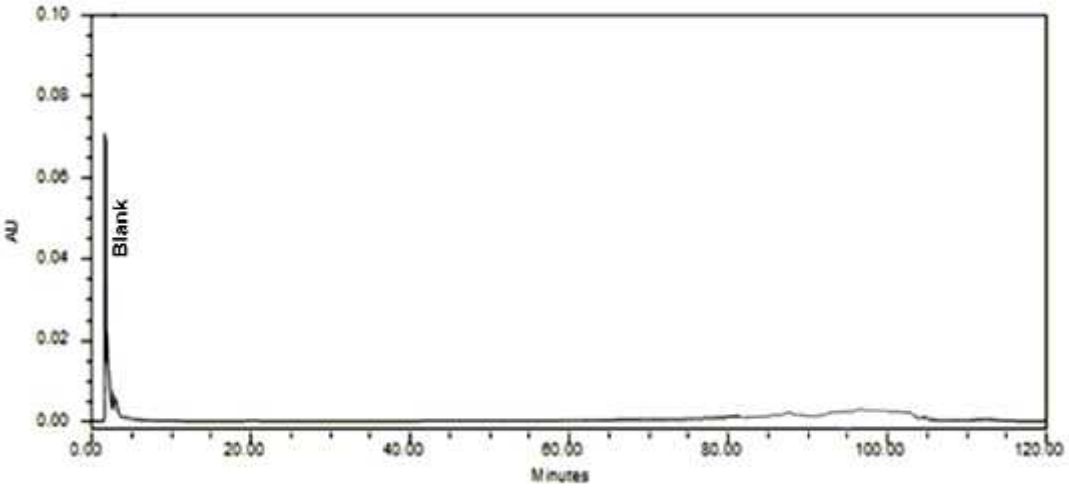


Figure 77: Chromatogram of 0.1 N NaOH stressed blank sample.

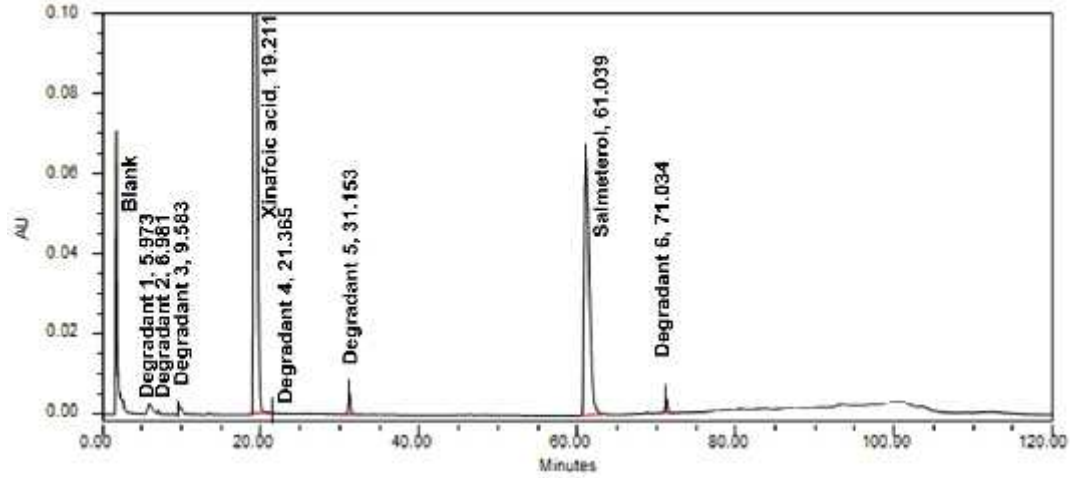


Figure 78: Chromatogram of 0.1 N NaOH stressed solution

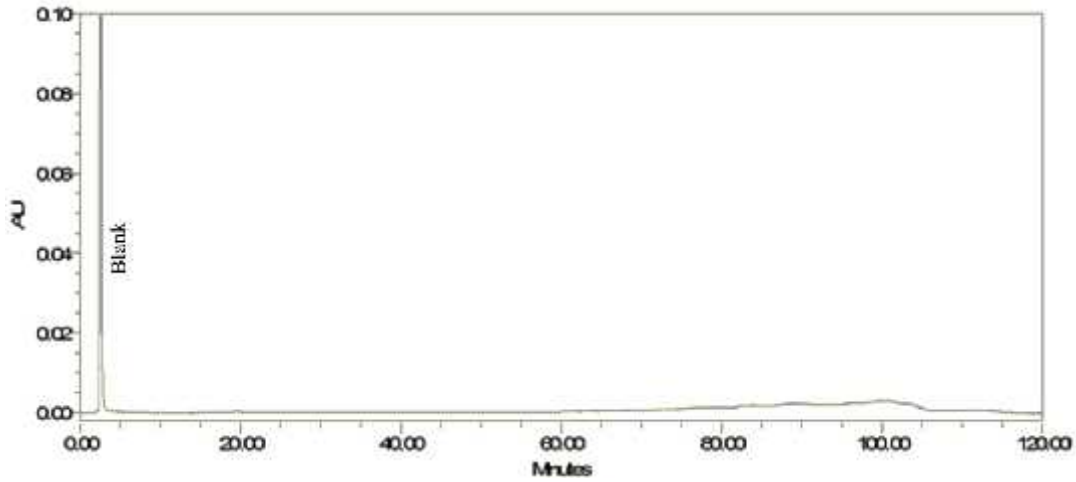


Figure 79: Chromatogram of 3% H_2O_2 stressed blank sample.

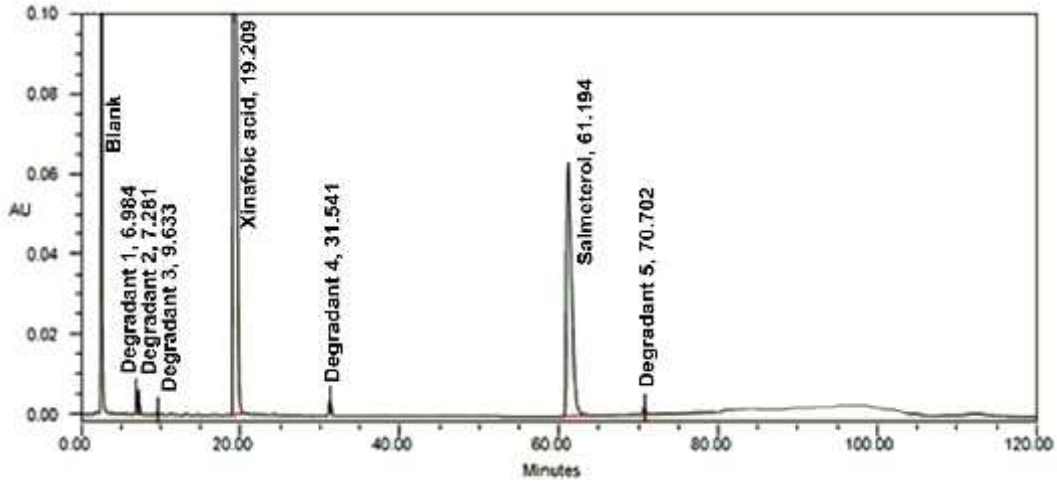


Figure 80: Chromatogram of 3% H_2O_2 stressed solution

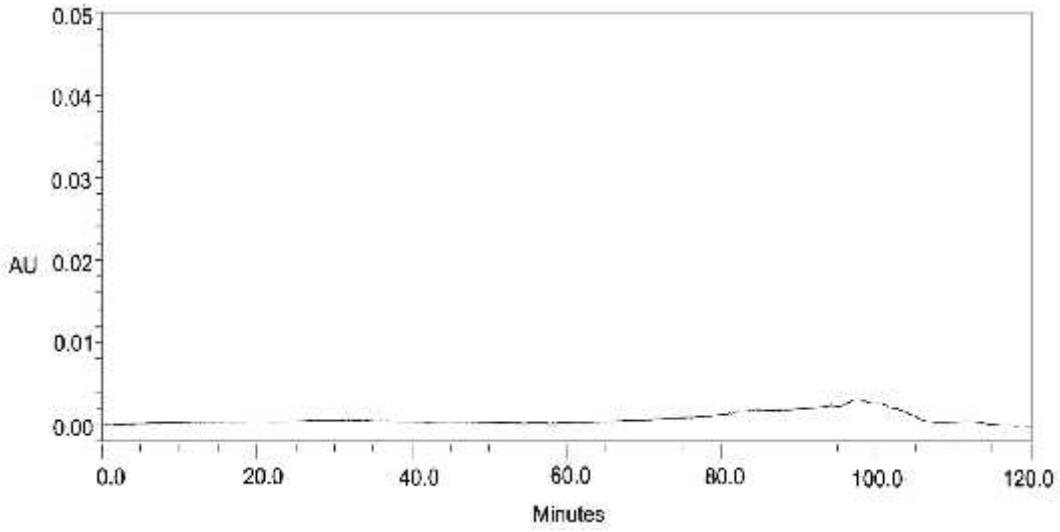


Figure 81**:** Chromatogram of thermal stressed blank sample

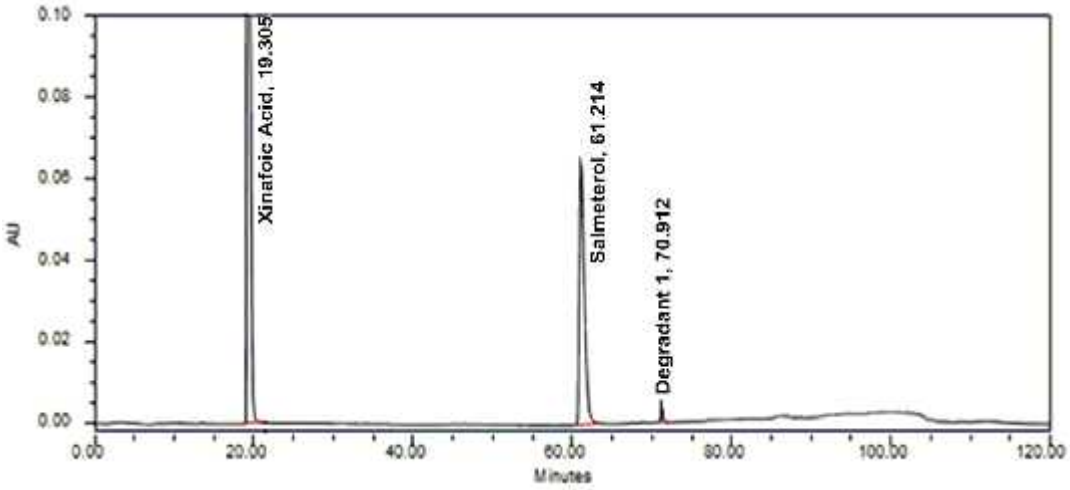


Figure 82**:** Chromatogram of thermal stressed sample

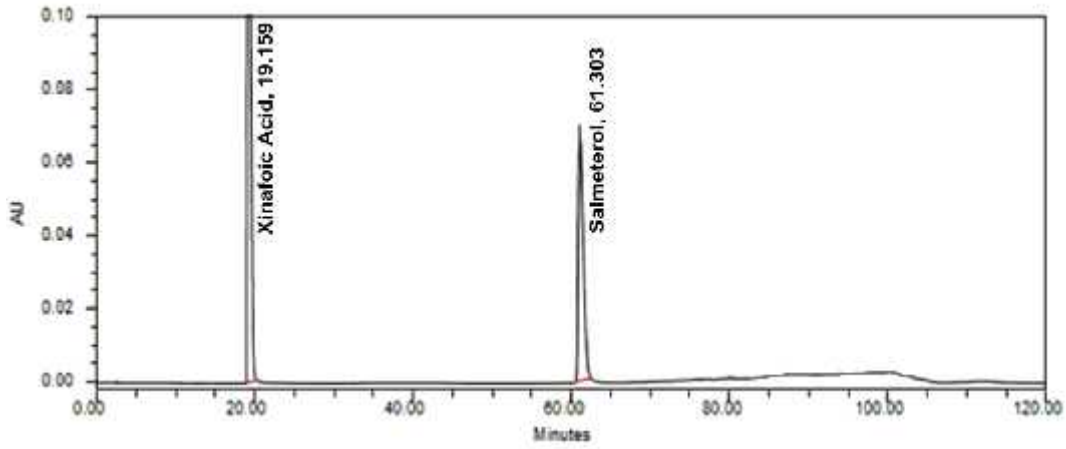


Figure 83**:** Chromatogram of light stressed sample

7.6 Conclusion

The method developed and validated for determination of salmeterol and its related substances in Inhaler dosage form by RP-HPLC is precise, linear, accurate, rugged and robust enough. The stability-indicating nature of the method was proven by peak purity testing of the forced degraded sample. Test sample solution was found to be stable up to 24 h at room temperature. Hence, this method can be considered valid for its intended purpose of establishing the quality of the drug product during product development and routine analysis.

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9.0 ABBREVIATIONS

RP-HPLC	Reverse phase-high performance liquid chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
ICH	International Conference on Harmonisation
ADME	Absorption, Distribution, Metabolism, and Excretion
FDA	Food and Drug Administration
pKa	Ionization constant
GC	Gas Chromatography
API	Active pharmaceutical Ingredient
FB	Free Base
DSC	Differential Scanning Calorimeter
QbD	Quality by Design
PDA	Photodiode Array Detector
%RSD	Percentage relative standard deviation
cDNA	Complementary Deoxyribonucleic acid
HLM	Human Liver Microsome
CYP2C8	Cytochrome P450 enzyme
DCQ	N-desethylchloroquine
ACF	Aceclofenac
LOD	Limit of Detection
LOQ	Limit of Quantitation
NSAID	Nonsteroidal anti-inflammatory drug
FB	Free Base
DSC	Differential scanning calorimeter
CNBQ	Cyclen Bisquinoline
LDH	Lactic Acid Dehydrogenase
PEG 400	Polyethylene glycol
PG	Propylene glycol
DMSO	Dimethyl sulfoxide
TLC	Thin-layer chromatography

NMR	Nuclear magnetic resonance
CDCl3	Deuterated chloroform
TMS	Tetramethylsilane