USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC ANALYSIS OF CIPROFLOXACIN TABLET AND INFUSION

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DECLARATION

It is hereby declared that this research was carried out by myself and has not been submitted, wholly or partially, for any other degree elsewhere. Any other assistance has been duly acknowledged.

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DEDICATION

This work is dedicated to my mother, Gladys Asiamah

ACKNOWLEGDMENT

I give thanks and praise to the almighty God for giving me the strength and endurance that kept me through this research.

To my supervisors, Mr. S. Asare-Nkansah, Mr. S. Oppong Bekoe and Mr. J. Oppong kyekyeku, I wish to express my profound gratitude. The knowledge and time they gave me is indeed priceless.

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I thank my family for the prayers, advice, support and love they showed me throughout the challenging times.

My gratitude also goes out to my colleagues for the support and encouragement. God bless you all.

ABSTRACT

In the search for an alternative means of analyzing Ciprofloxacin tablet and infusion, a simple and rapid high performance liquid chromatographic method was developed. This procedure involved the use of Benzoic acid and Salicylic acid as surrogate reference standards. The assay of Ciprofloxacin tablet and infusion employed a C-18 phenomenex 300 x 3.9mm reverse phase column with an isocratic mixture of methanol: water: phosphate buffer ,of pH-3 ,(50:45:5) at a flow rate of 1.5ml/min. The analysis was carried out at a wavelength of 239nm to provide good separation and intensity of the peaks for each compound. The mean retention times of Ciprofloxacin, Benzoic acid and Salicylic acid were **2.68±0.20**, **4.44±0.15** and **4.69±0.15** minutes. When Benzoic acid was used as a surrogate standard the constant K obtained was **0.3921±0.008**, with percentage contents of **99.99±0.53**, **95.90±0.48**, **97.85±0.62**, **99.06±0.64** and **96.79±0.62** for the five brands of Ciprofloxacin tablets. Similarly when Salicylic acid was employed as a surrogate reference standard the k value obtained was **0.4602±0.010** yielding percentage contents of **100.35±0.34**, **96.90±0.19**, **96.50±0.27**, **97.81±0.50** and **95.83±0.68** for the different brands of tablets. In the assay of the Ciprofloxacin infusions using Benzoic acid as surrogate standard percentage contents of **96.89±0.53**, **100.17±0.68**, **95.58±0.37** and **95.60±0.38** were obtained whereas salicylic acid as surrogate standard gave **96.64±0.58**, **100.04±0.52**, **96.54±0.36** and **97.37 ±0.29**. The results obtained from the developed method were statistically comparable with the standard method except for two brands. The method showed adequate precision, with relative standard deviations less than 2% in each case.

The method developed in this study was robust and linear within the working range and could be used for the quantitative analyses of Ciprofloxacin tablets and infusions.

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CHAPTER ONE

1.0 INTRODUCTION

Mikhail Tsweet, a Russian scientist, first used chromatography in 1906 to separate plant pigments such as chlorophyll and xanthophyll. This was done by passing the sample through a glass column packed with calcium carbonate. Chromatography is a technique which separates components in a mixture due to the difference in time taken for each component to travel through a stationary phase when carried along by a mobile phase. [1]

High Performance Liquid Chromatography (HPLC) is a mode of chromatography that is now mostly used as an analytical technique in the chemical laboratory. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixture. [2]

HPLC has been used in various analyses running through pharmaceuticals, food, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

In the pharmaceutical industry HPLC-based techniques are well accepted methods. It is a powerful technology that allows complex mixtures to be transformed into separated components. HPLC as compared with the classical Liquid Chromatography technique is characterised by: high resolution, small diameter (4.6 mm), stainless steel, glass or titanium columns, packing with very small (3, 5 and 10 μm) particles, relatively high inlet pressures and controlled flow of the mobile phase, continuous flow detectors capable of handling small flow rates and detecting very small amounts, rapid analysis. [3]

Perhaps the output from the HPLC is its unique characteristic that distinguishes it from all other analytical techniques. The chromatogram, which is obtained after each run, is defined and simple. Each peak is specific of a component; each chromatogram is diagnostic of an event or experiment associated with a drug development activity. Notably, most compounds of pharmaceutical interest are amenable to HPLC methodologies and conditions, and that critical information on nearly all events in the drug development cycle can be derived from HPLC chromatograms. [4]

There are many ways to classify liquid column chromatography. However, if this classification is based on the nature of the stationary phase and the separation process, three modes can be specified. In Adsorption chromatography the stationary phase is an adsorbent, like silica gel or any other silica based packing, and the separation is based on repeated adsorption-desorption steps.

For an Ion-exchange chromatography the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or

ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

In size exclusion chromatography: the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the pores of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography.

Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is nonpolar (such as n-hexane). Polar samples are thus retained on the polar surface of the column packing for longer than less polar materials.

Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run. [5]

In HPLC, the type and composition of the eluent is one of the variables influencing the separation. Despite the large variety of solvents used in HPLC, there are several common

properties: purity, detector compatibility, solubility of the sample, low viscosity and chemical inertness.

HPLC requires a sample preparation, analysis of the sample and interpretation of the results through calculation. It is therefore very important that the compounds involved are thoroughly analysed to ascertain their purity. This enables the analyst to ascertain the performance of the method for every component involved. [6]

Analysis in HPLC requires an internal standard. The internal standard is added to samples in known amount and carried through the procedure as a reference for calibration and controlling instrumental and analytical precision and bias. The standard should have chemical characteristics similar to those of analyte. Thus it provides analytical response that is distinct from the analyte and not subject to interference. Added to the sample immediately prior to analyses internal standards are used to adjust for variations in analytical response due to instrumental and/or matrix effects and, variations in the amount of sample provided for analyses due to variable injection volumes.

The internal reference sample is normally a pure sample of the analyte and when this pure sample is not available for HPLC analysis a surrogate reference standard can be used. A Compound that is used in analysing another compound due to similarity in properties to the analyte with reference to a specific analytical method that can identify and quantify the analyte is called a surrogate. Some factors that may be considered before choosing a compound as a surrogate include similarity in molecular structure, chemical stability and ability of the analytical tool to detect the compound. [6]

1.1 JUSTIFICATION

In HPLC analysis reference samples are needed for quality assessment and quantitative determination of the amount of compound present. The reference standard is required for calibration, validation of the method, quality control and quality assurance in general. As such pure reference standards are needed.

In this kind of HPLC analysis a reference standard is employed to draw a calibration curve for subsequent determination of concentration. However, the cost of Ciprofloxacin Hydrochloride reference standard, at $204.00 for 1mg, is expensive considering the economics of the country. Acquisition of these reference standards is scarce since the demand exceeds supply in terms of availability. Also to be considered is the cost of importation. The reference standard is costly considering all these factors in totality. In addition the length of time taken for the purchase, shipping and delivery also militates against the readily availability of the standard for any work.

As such there are times when the reference standard is difficult to come by in the research field or the industry. Quality monitoring of the drug becomes a challenge. This can also go a long way to have adverse effect on drugs produced.

Pure reference sample may not be available for all drugs and as such alternative methods need to be developed in such situations. This research therefore seeks to develop an alternative analytical procedure that would make it possible to use quantitative HPLC for assays without using pure reference sample of the target analyte, but chemically related compound known as surrogate reference standard.

Table 1: Cost of Ciprofloxacin pure reference sample

Catalog no	Product description	Current lot	Unit price
1134335	Ciprifloxacin (400mg)	JOH307	$204.00 Each

Source: USP Daily Reference Standards Catalog

1.2 MAIN OBJECTIVE

This project seeks to investigate the possibility of using surrogate reference standards for the analysis of ciprofloxacin tablets and infusion, by using HPLC.

1.2.1SPECIFIC OBJECTIVES

Specific objectives of this project are to

- Develop an HPLC assay procedure for Ciprofloxacin tablets and infusions using surrogate reference compounds.
- Validate the method developed by using validation parameters such as linearity, precision, accuracy, Limit of detection (LOD), Limit of quantification (LOQ).
- Determine a constant, K that can effectively be used for quantitative analysis of Ciprofloxacin tablets and infusions.
- Determine the percentage content of Ciprofloxacin in various brands of tablets and infusions using the method developed.
- To compare the results obtained from the method developed with a standard pharmacopoeial method.

1.3 HYPOTHESIS OF THE STUDY

The peak area, A, in a chromatogram is directly proportional to the concentration of the analyte, C. Implies A α C

Introducing a constant, K

A = KC , K = A/C. For similar compounds, the constant remains the same

$$\frac{A_{analyte}}{C_{analyte}} = \frac{A_{standard}}{C_{standard}}$$

$A_{analyte}$ is the peak area of the analyte

$A_{standard}$ is the peak area of the standard

$C_{standard}$ is the concentration of the standard

$C_{analyte}$ is the concentration of the analyte

Using surrogate compound as standard

$$\frac{A_{analyte}}{C_{analyte}} \neq \frac{A_{standard}}{C_{standard}}$$

But $\frac{A_{analyte}}{C_{analyte}} \propto \frac{A_{standard}}{C_{standard}}$

Introducing a constant, K, $\frac{A_{analyte}}{C_{analyte}} = K \frac{A_{standard}}{C_{standard}}$

,where K is a constant dependent upon the nature of surrogate compounds in relation to the analyte.

Once K has been obtained from the method developed, the $A_{analyte}$ and $A_{standard}$ are calculated from the chromatogram and $C_{analyte}$ can be calculated.

Thus $C_{analyte} = \frac{A_{analyte}}{A_{standard}} \times \frac{C_{standard}}{K}$

Percentage concentration= $\frac{\text{Actual concentration}}{\text{Nominal concentration}} \times 100$

CHAPTER TWO

2.0 LITERATURE REVIEW

2.0.1 Analyte and Surrogate reference standards profile

2.0.1.1 Ciprofloxacin hydrochloride

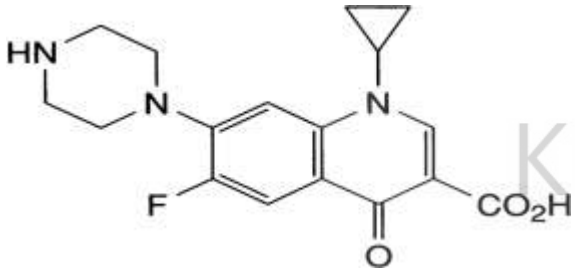

Figure 1: Chemical structure of Ciprofloxacin $C_{17}H_{18}FN_3O_3$

After the discovery of nalidixic acid in 1962 by Lesher et al., efforts have been made to modify the quinoline nucleus in other to increase antimicrobial activity and improve pharmacokinetic performance. A significant discovery was the fact that a fluorine atom at position 6 conferred broad and potent antimicrobial activity. However, this came with relatively less activity for gram-positive and anaerobic organisms than gram-negative bacteria. Further research developed quinolones with further improvements, predominantly in solubility (e.g. ofloxacin), antimicrobial activity (e.g. ciprofloxacin) or prolonged serum half-life. Ciprofloxacin is the most commonly used fluoroquinolones and it is a broad-spectrum antibiotic, effective against both gram positive and gram negative organisms.[7]

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperazin-1-yl)-quinoline -3-carboxylic acid) is a broad spectrum antibacterial drug to which most Gram-negative bacteria are highly susceptible *in vitro* and many Gram-positive bacteria are susceptible or moderately

susceptible. The primary mechanism of action of ciprofloxacin is inhibition of bacterial DNA gyrase.[7], [8],

Ciprofloxacin is presented as intravenous infusion and tablet. Ciprofloxacin is white or pale yellow, crystalline powder that is slightly hygroscopic. Ciprofloxacin is practically insoluble in water but slightly soluble in ethanol and methylene chloride with a melting point range of 255-257^{o}C. [9][10]

2.0.1.2 Salicylic acid

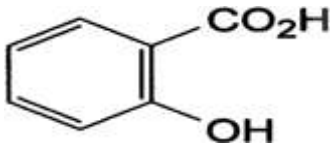

Figure 2: chemical structure of salicylic acid $C_7H_6O_3$

Salicylic acid, also known as 2-hydroxybenzenecarboxylic acid, is keratolytic and it is probably best known for its use in anti-acne treatments. Salicylic acid as an analgesic is used to ease aches and pains and reduce fevers. These properties of salicylic acid, particularly fever relief, have been known since ancient times, and it is used as an anti-inflammatory drug.

The preparations of salicylic acid come in the form of ointment and paste. It is also prepared as a collodion. [9], [11]

Salicylic acid is Colourless, feathery crystals or a white crystalline powder with melting point 158 – 161^{o}C . It is soluble in ethanol and ether, sparingly soluble in chloroform and slightly soluble in water. [10]

Salicylic acid is rapidly absorbed and distributed throughout the body and happens to be the major metabolite of aspirin. It is metabolized by conjugation with glucuronic acid and glycine to give salicyluric acid, salicyl *O*-glucuronide, and salicyl ester glucuronide. Aspirin or

acetylsalicylic acid can be prepared by the esterification of the phenolic hydroxyl group of salicylic acid with the acetyl group from acetic anhydride or acetyl chloride. Thus salicylic acid can be prepared by hydrolysis of aspirin or methyl sailcylate with a strong acid. [12]

2.0.1.3 Benzoic acid

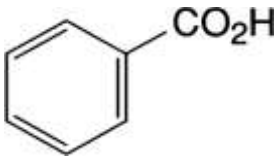

Figure 3: Chemical structure of benzoic acid $C_7H_6O_2$

Benzoic acid, otherwise known as benzenecarboxylic acid or phenylformic acid, is used as an antimicrobial preservative in medicines and food that comes as an ointment or solution. [9] Benzoic acid is used in many cosmetics with the derivatives involved in the dyeing industry. Pure benzoic acid is a standard for bomb calorimetry because of its ease of purification by sublimation. In combination with salicylic acid, it is used in the form of an ointment for treating fungal infections of the skin, such as ringworm; it is also included in some soothing preparations for haemorrhoids. Benzoic acid also has keratolytic properties and is used in combination with malic acid and salicylic acid for removing dead skin from ulcers, burns, and wounds. [13] [14]

Benzoic acid is Colourless, light feathery crystals or white scales or powder with a melting point range of 121-124^{o}C and sublimes after heating. It is freely soluble in acetone, soluble in ethanol, ether and chloroform but slightly soluble in water.

Benzoic acid is prepared in the laboratory by the Grignard reaction, hydrolysis of benzonitrile (C_6H_5CN), or prolonged oxidation of alkyl benzenes with potassium permanganate regardless of the length of the alkyl group.

2.0.2 INSTRUMENTATION OF ANALYTICAL TECHNIQUES

2.0.2.1 ULTRAVIOLET –VISIBLE SPECTROSCOPY

Spectroscopic methods of analysis are procedures used to determine the amount of substance in analytes using the quantity of radiation absorbed or emitted by the atoms or molecules. [15] In ultraviolet –visible spectroscopy compounds are analysed based on their absorbance or reflectance within the ultraviolet –visible region of the electromagnetic spectrum. In this instance molecules undergo transition from the ground state to the excited state when they absorb the radiation. The more easily excited the electrons the longer the wavelength of light it can absorb.

The Beer-Lambert law, which is the basis for this technique, states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length.[16] Hence, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorbing specie in a solution, as such the absorbance changes with concentration. This can be determined more accurately from a calibration curve. The UV-Vis spectrophotometer may be used as a detector for HPLC Because of this property. The analyte gives a response, which is due to absorbance by the analyte concentration. For the purpose of accuracy, the instrument's response for the analyte in the unknown can be compared with the response to a standard. The response (e.g., peak height) for a particular concentration is known as the response factor. [17]

The wavelengths of absorption peaks correlate with the types of bonds in a given molecule and are used in determining the functional groups within a molecule. Factors such as the nature of solvent, pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances all affect the absorption spectrum. Thus these variables must be controlled

in identifying the substances present when using the UV-Vis spectroscopy for analysis.[18] From the Beer-Lambert law absorbance can be expressed as:

$$A = - \log_{10} (I / I_o) = \varepsilon c L$$

where A is the measured absorbance, I_o is the intensity of the incident light at a given wavelength, I is the transmitted intensity, L the pathlength through the sample, and c the concentration of the absorbing species. For each species and wavelength, ε is a constant known as the molar absorptivity or extinction coefficient. This constant is a fundamental molecular property in a given solvent, at a particular temperature and pressure.

The ratio I / I_o is called the transmittance, and is usually expressed as a percentage (%T). The absorbance is based on the transmittance:

$$A = -\log (\%T / 100\%)$$

The UV-visible spectrophotometer can also be configured to measure reflectance. In this case, the spectrophotometer measures the intensity of light reflected from a sample, I, and compares it to the intensity of light reflected from a reference material, I_o, such as a white tile. The ratio I/I_o is called the reflectance, and is usually expressed as a percentage (%R).

A spectrophotometer can be either single beam or double beam. In a single beam instrument, all of the light passes through the sample cell. I_o must be measured by removing the sample. This was the earliest design and is still in common use in both teaching and industrial labs. In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample. The reference beam intensity

is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities.

In UV-Vis spectrophotometry, most samples are often liquids, although the absorbance of gases and even of solids can also be measured. Samples are placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1 cm, which is the path length, L, in the Beer-Lambert law. The type of sample container used must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of fused silica or quartz because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths.[19]

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecule.

2.0.2.2 Thin Layer Chromatography

Thin layer chromatography is the most widely used chromatographic method for qualitative analysis of mixtures. It involves a mobile phase which moves by capillary action on a stationary phase. [20] Thin layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents. [21]

2.0.2.2.1 Stationary phase

Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, cellulose and cellulose derivatives, ion-exchange resins, kieselguhr, or polyamides on a flat, inert substrate or plate (blotter paper). This layer of adsorbent is known as the stationary phase. The adsorbent could contain a binder of calcium sulphate (15%) and a fluorescence indicator such as fluorescein (15%). This is to allow for easy detection of compounds that quench the fluorescence upon observing in UV light. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used. [20], [22]

2.0.2.2.2 Mobile phase

When a sample is applied on the plate, a solvent or solvent mixture known as the mobile phase is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. [22]

2.0.2.2.3 Separation procedure

Separation of the various components of a mixture in thin layer chromatography is achieved by the differences in solubility in the solvent and the differences in their attraction to the stationary phase ,as such these components travel at different rates. Changing the solvent or using a mixture can adjust the separation of components. [23]

In reality the separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. For instance, if normal phase silica gel is used as the stationary phase it can be considered polar. Given two compounds which differ in polarity,

the more polar compound has a stronger interaction with the silica and is therefore more capable to dispel the mobile phase from the binding places. Consequently, the less polar compound moves higher up the plate resulting in a higher Rf value. If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places and all compounds on the TLC plate will move higher up the plate. The order of strength/weakness depends on the coating on the stationary phase of the TLC plate. For silica gel coated TLC plates, the elutant strength increases in the following order: Perfluoroalkane, Hexane, Petroleum Ether, Pentane, Carbon tetrachloride, Benzene/Toluene, Dichloromethane, Diethyl ether, Chloroform, Ethyl acetate, Acetonitrile, Acetone, 2-Propanol/n-Butanol, Methanol, Water, Triethylamine, Acetic acid, Formic acid. The reverse holds for C18 coated plates. [21] [24]

2.0.2.2.4 Analysis

Several methods exist to visualize the spots. Usually a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under UV light at 254nm. The adsorbent layer will thus fluoresce light green by itself, but spots of analyte quench this fluorescence.

Iodine vapours' are a general unspecific colour reagent but Potassium permanganate, Iodine or Bromine could be used as specific colour reagents into which the TLC plate is dipped or sprayed onto the plate. [25]

For lipids, the chromatogram may be transferred to a polyvinylidene difluoride membrane and then subjected to further analysis, for example mass spectrometry, a technique known as Far-Eastern blotting. [26]

The retardation factor, R_f of each spot can then be determined by dividing the distance the product travelled by the distance the solvent front travelled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants. [27]

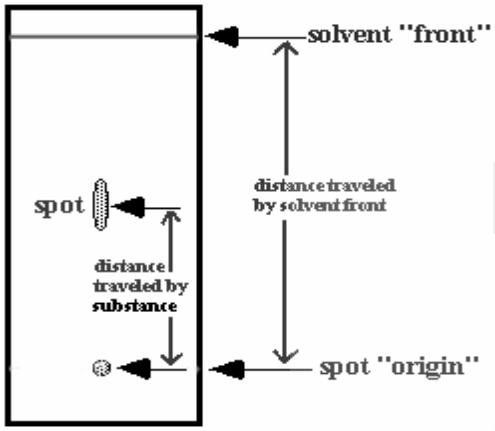

Figure 4: TLC plate showing the distances travelled by the spot and the solvent.

2.0.2.3 Acid-base titrations

An acid-base titration is the determination of the concentration of an acid or base by exactly neutralizing the acid/base with an acid or base of known concentration. This allows for quantitative analysis of the concentration of an unknown acid or base solution. It makes use of the neutralization reaction that occurs between acids and bases and the knowledge of how acids and bases will react if their formulas are known. Acid–base titrations can also be used to find percent purity of chemicals.

One of the most important aspects of acid-base titrations is the choice of indicator. At the equivalence point, the point at which equivalent amounts of the reactants have reacted, the solution will have a pH dependent on the relative strengths of the acid and base used. Generally a

strong acid will react with a strong base to form a neutral (pH=7) solution whilst strong acid will react with a weak base to form an acidic (pH<7) solution. Then weak acid will react with a strong base to form a basic (pH>7) solution. However, weak acids are not often titrated against weak bases because the colour change shown with the indicator is often quick, and therefore very difficult for the observer to see the change of colour.

Acid–base titration is performed with a phenolphthalein indicator, when it is a strong acid – strong base titration, a bromthymol blue indicator in weak acid – weak base reactions, and a methyl orange indicator for strong acid – weak base reactions. If the base is off the scale, i.e. a pH of >13.5, and the acid has a pH >5.5, then an Alizarine yellow indicator may be used. On the other hand, if the acid is off the scale, i.e. a pH of <0.5, and the base has a pH <8.5, then a Thymol Blue indicator may be used. [28]

2.0.2.4 Non-Aqueous titrations

Compounds that are weakly basic or weakly acidic to give sharp end-points in aqueous solvents may be analysed in non-aqueous solvents. This is because they acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by non-aqueous titration. To prevent interference from excipients it is always important to isolate the active ingredient in the analysis of pharmaceutical preparations. [29]

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. On the contrary, compounds that may be titrated as bases include

amines, nitrogen-containing heterocyclic compounds, quarternary ammonium compounds, alkali salts of organic acids, alkali salts of inorganic acids, and some salts of amines.

The most commonly used procedure is the titration of organic bases with perchloric acid in anhydrous acetic acid. Acetic acid an amphiprotic solvent functions as a base in the presence of perchloric acid. As such the stronger onium ion that is formed, donates proton to the base whereas the solution of perchloric acid in glacial acetic acid acts as a strong acid.

$HClO_4 \rightarrow H^+ + ClO_4^-$

$CH_3COOH + H^+ \rightleftharpoons CH_3COOH_2^+$ (onium ion)

$HClO_4 + CH_3COOH \rightleftharpoons CH_3COOH_2^+ + ClO_4^-$

$R_3N + CH_3COOH \rightleftharpoons R_3NH^+ + CH_3COO^-$

$CH_3COOH_2^+ + CH_3COO^- \rightarrow 2CH_3COOH$

Adding $HClO_4 + R_3N \rightleftharpoons R_3NH^+ + ClO_4^-$

Salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex. In the case of hydrochlorides of weak bases not containing acetyl groups it is also possible to titrate in acetic anhydride without the addition of mercuric acetate and using an indicator such as malachite green or crystal violet. Titrations carried out in the presence of an excess of acetic anhydride must be applied cautiously, however, since any reaction of the anhydride with the substance being titrated may give rise to low results. The end-point may be determined visually by colour change, or potentiometrically. [21][29][30]

2.0.2.5 High Performance Liquid Chromatography (HPLC)

2.0.2.5.1 Introduction

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. High performance Liquid Chromatography is used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. Some common examples are the separation and quantitation of performance enhancement drugs.

HPLC makes use of different types of stationary phases contained in columns, a pump that moves the mobile phase and sample components through the column at, and a detector capable of providing characteristic retention times for the sample components, with area corresponding to the amount of each analyte passing through the detector. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive. [21][1]

2.0.2.5.2 Theory of HPLC

In High Performance Liquid Chromatography, a pump, rather than gravity, provides a high pressure to move the mobile phase and sample components through the densely packed column. The increased density arises from the use of smaller sorbent particles. Such particles are capable of providing better separation on columns of shorter length when compared to ordinary column chromatography. The amount of resolution is important and dependent upon the extent of interaction between the solute components and the stationary phase. This is enhanced by the fact that it allows you to use a very much smaller particle size for the column packing material which

gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it, giving a much better separation of the components of the mixture compared to liquid chromatography.

2.0.2.5.3 The stationary phase

The stationary phase can be a liquid or solid material and it is the immobile packing material in the column. HPLC separations are highly dependent on the surface interactions and adsorption site. Modern HPLC adsorbents are small rigid porous particles with high surface area. Usually the particle-size of the various columns is between 3 to 10μm, narrowly distributed. Depending on the type of the ligand attached to the surface the adsorbent could be normal phase (-OH, -NH_2), or reversed phase (C_5, C_8, C_{18}) and even anion exchangers ($CH_2NR_3^+OH^-$) or cation exchangers ($R\text{-}SO_3^-H^+$).

2.0.2.5.4 The Mobile Phase

The analyte is introduced, in a discrete small volume, into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities depending on its chemical nature, the nature of the stationary phase and on the composition of the mobile phase. The time at which a specific analyte emerges from the column is called the retention time. The use of smaller particle-size packing materials requires the use of higher operational pressure which improves the degree of separation between consecutive analytes emerging from the column. [1]

Common mobile phases used include any miscible combination of water with various organic solvents. The aqueous component of the mobile phase may also contain buffers, acids or salts to assist in the separation of the sample components. Meanwhile, the composition of the mobile phase could be maintained or varied during the chromatographic analysis. The composition of

the mobile phase depends on the intensity of interactions between analytes and stationary phase. As a result of the affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column.

2.0.2.5.5 Modes of Chromatographic Separations

The modes of chromatography differ in terms of the nature, composition and structure of the stationary phase, and in the nature of the molecular forces that hold the solute molecules within the mobile and stationary zones. [31]

2.0.2.5.5.1 Partition chromatography

Partition chromatography, the first kind of chromatography that was developed utilizes a retained solvent, on the surface or within the grains or fibers of an inert solid supporting matrix. During analysis, equilibrium for the constituents is set between the liquid mobile phase and the bonded stationary phase. The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. This phenomenon is also known as Hydrophilic Interaction Chromatography (HILIC) in HPLC. The method separates analytes based on polar differences. In partition chromatography a bonded polar stationary phase and water miscible, high organic concentration, mobile phases are employed. Quite a number of bond phases are available commercially. Partition HPLC has been used historically on unbonded silica or alumina supports. These provide a range of techniques that can enable separation of a wide variety of mixtures.

In Partition Chromatography retention strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time. The interaction strength depends on the functional groups in the analyte molecule which promote

partitioning but can also include electrostatic interaction and hydrogen donor capability. In effect the use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times. [32][35]

2.0.2.5.5.2 Adsorption chromatography

Adsorption chromatography also known as normal-phase HPLC separates analytes based on their affinity for a polar stationary surface such as silica or alumina, hence it is based on analyte ability to engage in polar interactions, such as hydrogen-bonding or dipole-dipole type of interactions, with the sorbent surface. NP-HPLC uses a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity. The interaction strength depends not only on the functional groups present in the structure of the analyte molecule, but also on steric factors. The effect of steric hindrance on interaction strength allows this method to separate structural isomers.

The use of more polar solvents in the mobile phase decreases the retention time of analytes, whereas more hydrophobic solvents tend to increase the retention times. Highly polar solvents in the mobile phase tend to adsorb to the solid surface of the stationary phase forming a stationary bound layer which is considered to play an active role in retention. It is as a result of this property that it is sometimes referred to as Normal Phase HPLC. Adsorption chromatography is widely used for structural isomer separations in both column and thin-layer chromatography formats on activated silica or alumina supports. [35]

2.0.2.5.5.3 Reverse Phase Chromatography

The use of non-polar bonded stationary phase with polar mobile phase was introduced because of poor reproducibility of retention times due to the presence of water or protic organic solvent layer on the surface of the silica or alumina chromatographic media in the Normal phase chromatography.

Reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. A typical stationary phase in this type is silica which has been modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is longer for less polar molecules, where as polar molecules elute more readily. An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase.. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release. [35]

RP-HPLC operates on the principle of hydrophobic interactions, which originate from the high symmetry in the dipolar water structure. The retention of an analyte to the stationary phase depends on the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. The retention can be decreased by the addition of a less polar solvent like methanol or acetonitrile to the mobile phase. The application of Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis. In general, an analyte with a larger hydrophobic surface area is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area, due to the presence of polar

groups like -OH, $-NH_2$, COO^- or $-NH_3^+$ in their structure, are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with the surface take place. Such surface hindrance typically results in less retention. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond. [33]

Another important factor is the mobile phase pH since it can change the hydrophobic character of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. Buffers serve multiple purposes: control of pH, neutralize the charge on the silica surface of the stationary phase and act as ion pairing agents to neutralize analyte charge. The effects of acids and buffers vary by application but generally improve chromatographic resolution.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should not be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment.

2.0.2.5.5.4 Ion exchange chromatography

Ion-exchange chromatography is also notable in pharmaceutical and biochemical analysis.In ion-exchange chromatography, separation is as a result of the attraction between solute ions and charged sites of the stationary phase. This results in the exchange of ionic analytes with counter-ions of the ionic groups attached to the solid support. Cation exchangers include NR_3^+, NH_3^+ where as some anion exchangers are SO_3^-, COO^- . Thus ions of the same charge are excluded. Types of stationary phases are: polystyrene based phases, acrylic polymer based phases, dextran and cellulose based phases and controlled-pore glass or porous silica.

An increase in counter ion concentration reduces the retention time. A decrease in pH reduces the retention time in cation exchange while an increase in pH reduces the retention time in anion exchange. By lowering the pH of the solvent in a cation exchange column, for instance, more hydrogen ions are available to compete for positions on the anionic stationary phase, thereby eluting weakly bound cations. [34]

Ion exchange chromatography is used in: water purification, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and others. [35][36]

2.0.2.5.5.5 Size-exclusion chromatography

Size-exclusion chromatography, also known as gel permeation chromatography or gel filtration chromatography separates particles based on their size. Normally, it is employed at final stages of separation for brushing up since it is a low resolution format. In this kind of chromatography smaller molecules are trapped in the pores of a particle of the stationary phase. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules

therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time. This technique is widely used for the molecular weight determination of polysaccharides.[34][35][36]

2.0.2.5.6 Quantitative and Qualitative Analysis in HPLC

The essence of qualitative analysis is to identify the unknown compound in a sample. Peak identification is done by comparing the retention times with those of a standard. Another useful technique involves spiking the sample with known amount.

The area of a peak, on the chromatogram is directly proportional to the amount of that particular component passing through the detector. This, in turn, depends on the concentration of that mixture component in the sample solution. It is also in relation to the amount of solution injected, since this too dictates how much passes through the detector. In effect more materials detected correspond to a larger peak. As such quantitative analysis requires that accurate method be designed for determining the areas of the peak.[37]

The most common means of measuring a peak area is by integration. In this method a series of digital values acquired by the data system as the peak traced are summed. The total is a number generated, which is given out by the data system as the peak area.

The modern analytical laboratory employing HPLC makes use of a data collection system and its software to acquire the data and display the chromatogram on a monitor. Quantitative analysis is then done with parameters such as retention time and the peak areas that are measured and displayed.

2.0.2.5.7 Instrumentation and Trends

The HPLC consist of solvent reservoir for holding the mobile phase, a high pressure pump used to pump the mobile phase through the column, a specially made injection device for introducing the analyte, a column for the actual separation of components, a detector for identifying the components, after elution, electronically and a data system used to retrieve and display the chromatogram.

2.0.2.5.7.1 Reservoir

The reservoir of an HPLC system is preferably made of glass because it is inert. Fitted with it is a cap to prevent particulate matter from entering the system, even though there is a vent to allow entry of air. The tip of the tube immersed in the reservoir is fitted with a coarse metal file which serves as a sinker to keep the tip well immersed in the mobile phase. Some specially designed reservoirs have the filter placed in a well at the bottom to keep it well immersed even when the content is low. Mobile phase is drawn from the reservoir by vacuum action. Air bubbles are likely to cause problems like poor pump performance and poor detector response. As a result degassing is done at routine by helium sparging, ultrasonic agitation or drawing of vacuum over the liquid surface.

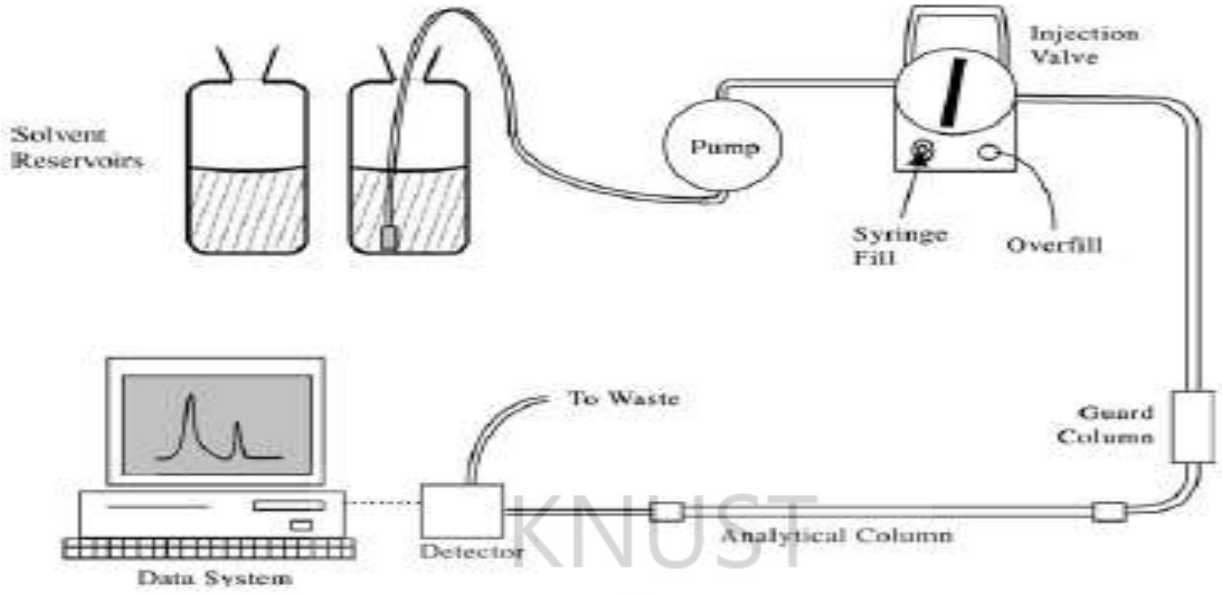

Figure 5: A graphic representation of an HPLC system.

2.0.2.5.7.2 Pump

An HPLC pump is expected to provide precise and pulse-free delivery of solvents at a rate of 0.1-10mL/min and pressure up to 42Mpa. Subsequently it is expected to be compatible with common organic solvents, buffers, salts, be reliable in its operation with long seal life and easy to maintain. Even though three types could be emphasised the most common is the reciprocating piston pump. This pump utilizes a motorized cam or direct screw-drive system that drives a piston back and forth to deliver solvent through inlet and outlet check valves. Only the inward stroke delivers liquid so a pulse dampener is used to reduce the rate. The reciprocating piston pump delivers solvent continuously, has no restriction on the reservoir size or operating time, can be varied in its flow rate, poses a quick and accurate change in solvent which is an advantage when doing gradient elution, and has a dual head and triple head pumps with identical piston-chamber units. Nevertheless the simple reciprocating pump design has been perfected through numerous innovations. The life of the piston seal was improved by better design coupled with front panel access to the pump heads for easier maintenance. The fluidic components in the

pumps for bio-purification are often made of titanium or polyetheretherketone to lesson corrosion by salt or metal ions. [37]

The syringe-type pumps operate through positive solvent displacement with piston that drives at constant rate mechanically. The properties of this pump are that: solvent delivery is controlled by changing the voltage on the digital stepping motor, the flow is without pulse, high pressure can be achieved, solvent chamber capacity is finite, and the solvent gradients are also possible through tandem operation of two or more pumps. Syringe-type pumps were popular in the 1960's because of their inherent precision and pulseless flow-rate. However, the cost of manufacture and problems associated with syringe refill cycles makes them rather useful in specialized systems.

In constant pressure pumps the mobile phase is driven by the pressure from a gas cylinder, which is delivered through a large piston. This pump has a low gas source i.e. 1 to 10 atm which can deliver the solvent up to pressures of 400atm, a large flow-rate for preparative applications, and flows without pulse and inconvenient for use in gradient elution. [34][36]

2.0.2.5.7.3 Injectors

The Injector for an HPLC is used to introduce the sample to the column at high pressure. Very common is the Rheodyne model. It has a six-port valve, sample loop and a needle port. A syringe with blunt tip needle is used to introduce a precise amount of the analyte into the sample loop. The sample is then pushed into the column by switching the valve from 'load' to 'inject' positions. For the purpose of reducing labour cost and increasing productivity an auto sampler can also be employed. This involves the use of the loop injector coupled to a robotic needle that draws samples from vials arranged in a carousel-type. [35][37][38]

2.0.2.5.7.4 Column

The HPLC column is the heart of the HPLC system. How fast a separation could be achieved depends primarily on the column length and particle size. Each type of HPLC utilizes a different separation mechanism and so the selection of a specific column depend s on whether the planned separation is possible or logical with a given mechanism. In general longer columns have a higher plate count. Embedded in the column is a fine support media for the stationary phase. This provides differential retention of sample components. Columns are constructed of heavy-wall, glass-lined metal from titanium, stainless steel, polyetheretherketone and filled with small particle packing's. Columns with internal diameter of 4 to 5mm are standard in HPLC with the particle diameter range between 4 to 7µm for most columns. The lengths also lie between 10 and 30cm. However, a short column i.e. 3 to 6cm is recommended for quick analysis.

The mode of chromatography could be used to classify a column. By support it could be silica, polymer, zirconia or hybrid type. In general the selection of a suitable HPLC should involve the consideration of components of the analyte such that the mixture must have a relatively high affinity for the stationary phase compared to the mobile phase, to ensure better resolution through considerable longer retentions.

It is noted that narrow bore columns offer increased detector signal for components, less solvent consumption, homogeneous packing density, better column permeability and better dissipation of frictional heat. Guard columns are recommended to act as chemical filter to remove particles or contaminants that could foul the column and consequently shorten its lifespan. The guard column is, ideally, packed with the same material as the analytical columns. [38]

2.0.2.5.7.5 Detector

The HPLC detector is responsible for examining the solution that elutes from the column and output an electronic signal which depends on the concentrations of individual components in the analyte. A recent survey indicates that85% of pharmaceutical applications use absorbance detectors such as UV/Vis detector or photodiode array detectors. [39]

2.0.2.5.7.5.1 UV/Vis Detector

The UV/Vis absorbance detector monitors the absorption of UV/Vis light in the HPLC eluent. They are mostly used because most analytes of pharmaceutical interest have UV absorbance. Most UV absorption bands correspond to transitions of electrons from the $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ or $n \rightarrow \sigma^*$.

The detector is made of a deuterium lamp, a monochromator that selects the wavelength, and a flow cell, which is like a cuvette, through which the column effluent flows. The monochromator consist of a movable grating or prism which permits the selection of selection of specific wavelength to pass through the exit slit. A dual beam optical design is what usually pertains. This involves a light source that splits into two, notably the sample and reference beam. The intensity of each beam is then monitored by a separate photodiode. The sample beam passes through the sample flow cell of volume lying between 2-10µL and path lengths of 2-10mm.The principle for UV/Vis absorption is also based on the Beer's Law, which mathematically is stated as Absorbance(A) = molar absorptivity (ε) x pathlength (b) x concentration (c) [40][46]

Two key advantages about this detection are the wavelength selectivity and its excellent analyte sensitivity. However, the mixture components should absorb light in the UV region in order for a

peak to appear on the recorder. The mobile phase must not also absorb an appreciable amount at the chosen wavelength.[40]

2.0.2.5.7.5.2 Diode Array Detector

The Diode Array detector gives UV spectra of the eluting peaks as well as the absorbance of the HPLC eluent like the UV/Vis absorbance detector. It is mostly preferred for testing impurities and method development. Light from the source passes through the flow cell onto a diode array element. The element measures the intensity of light at each wavelength. With the aid of the data system , the entire UV absorption spectrum can be measured as each individual component is eluted. The peaks displayed can represent the maximum possible sensitivity for each component. The diode array detector can be used to modify a chromatogram so that only the peaks of interest bare displayed. This is made possible by the rapid change in the wavelength giving rise to the peaks. Sensitivity of this detector intially was an issue, but has improved over a ten fold in recent years. [35]

2.0.2.5.7.5.3 Fluorescence Detector

Fluorescence detection for components could be highly sensitive than UV and selective for fluorescent compounds. Thus this procedure is recommended for trace analysis and complex matrices. Meanwhile, its application in pharmaceutical analysis is limited since few analytes of interest have strong innate fluorescence. The fluorescence detector monitors the emitted fluorescent light of an eluent in the HPLC flow cell with irradiation of an exciting light at right angle. The set up may consist of a regular fluorescence spectrometer with a flow cell. Fluorescence detection is constructed from xenon source, an excitation monochromator, an emission monochromator, a square flow cell and a photomultiplier. Light from the lamp source

passes through an excitation filter, for the provision of light of the desired wavelength, to excite the molecules of the sample. As the exciting light passes through the effluent in the flow cell it causes the molecules to emit at a higher wavelength than that used for excitation. The second filter collects light at 90^{o} to the actual direction of excitation. As a result only light coming from the sample fluorescence is passed onto the photomultiplier for quantification of the signals.[41][42]

2.0.2.5.7.5.4 Refractive Index

The refractive index detector measures the difference in refractive index between the cell containing the eluting analyte and the cell containing pure the eluent. It is a form of universal detection but low in terms of sensitivity (0.01-0.1g) and more prone to temperature and flow changes compared to UV/Vis absorbance. It is normally used for the analysis of low chromophoric activities such as sugars, triglycerides, organic acids, pharmaceutical excipients and polymers. Modern refractive index detectors are differential deflection type with thermostated flow cell. Thus they have improved baseline stability but their comparably low sensitivity and incompatibility with gradient elution is of much concern.[42]

2.0.2.5.7.5.5 Electrochemical Detector

An electrochemical detector works by measuring the current or conductivity associated with electroactive analytes in the HPLC eluent between the electrodes placed in the flow cell. It is sensitive for neurotransmitters, sugars, glycoprotein's, and compounds containing phenol, hydroxyl, amino, diazo or nitro functional groups. The detector could be one for amperometry or conductivity. This is very useful for ion exchange or chromatography in which the analyte is in ionic form. From fundamental science solutions containing ions give low electrical resistance. Thus if a pair of conductive surfaces are immersed in such a solution and connected to a power

source current flown is detected. The conductivity detector is based on this principle. In such a detector electrically isolated inlet and outlet tubes are used as electrodes. [37] [43]

The basis for the amperometric detector is oxidation-reduction of eluting mixture components. All the three electrodes required for the detection, i.e. indicator, reference and auxiliary electrodes are either inserted in the flow stream or placed in the wall of the flow stream. The indicator electrode causes oxidation of the mixture as elution occurs. The current associated is identified and sent to the recorder or integrator. It can be applied to both ionic and molecular components. The sensitivity of this detector is better than the UV detector but not the fluorescence detector. The disadvantage is that indicator electrode can be fouled due to coating of the electrode surface. [37]

2.0.2.5.7.5.6 Mass spectrometry Detection (MS)

Mass spectrometry is becoming very common in recent analysis of compounds even though it is costly and needs skilled operator. This mode of separation combines the versatility of HPLC with the identification strength of mass spectrometry. The challenge involved with this format has to do with the interface connecting the liquid stream at atmospheric pressure to the high vacuum in the mass spectrometer. It is highly recommended for bioresearch, drug discovery, combinatorial analysis and pharmacokinetic assays. MS has seen a lot of improvement with the introduction of several atmospheric pressure interfaces, electrospray and atmospheric pressure chemical ionization. [44]

2.0.2.5.7.5.7 Radioactivity Detector

The radioactivity detector measures the radioactivity in the eluent from the HPLC column with the making use of the flow cell. This is based on the detection of phosphors, caused by

radioactive nuclides, using liquid scintilator. The detector is specific only for radioactive compounds and could be really sensitive. It has found its usage in toxicological, metabolism and degradation studies. [35][39]

2.0.2.5.7.5.8 Other Detectors

Coupled with the separation of mixtures, elucidation of molecules has contributed to the introduction of new detectors. Some of which include NMR detector, chemiluminescence Nitrogen Detector, Corona-Charged Aerosol Detector, Evaporating Light Scattering Detector, IR Detector, Optical Activity Detector and Low-angle laser light scattering.

2.0.2.5.8 Uses of HPLC in Pharmaceutical Analysis

Pharmaceutical products are expected to be of the right quality in other to guarantee efficacy, safety and effective clinical outcome. In view of this, regulatory bodies provide specifications that must be met before a drug is marketed. The primary focus is to confirm the identity, purity and potency of drug substances. HPLC has assumed the most popular separation technique for analysing drug substances and products. Most assay and impurity testing on drug substances are conducted with HPLC. For drug products, HPLC is highly recommended for testing the bioavailability and used to ensure that the content of a drug substance is uniform throughout a batch. In other to preserve drug products from degradation, due to microbes and oxidation, generic HPLC assays are developed for monitoring preservatives. [45][46]

Many improvements have been made in HPLC instruments and software surrounding its operation to the effect that some labs run analyses overnight and even 24 hours unattended.

CHAPTER THREE

3.0 MATERIALS, REAGENTS AND METHODS

3.0.1 Materials / Reagents

Perchloric acid 70%, A.C.S (Aldrich)

Anhydrous acetic acid (BDH)

Acetic anhydride (BDH)

Potassium dihydrogen orthophosphate (BDH)

Hydrochloric acid, 32% (BDH)

Sodium hydroxide (BDH)

Acetonitrile (BDH)

Potassium hydrogen phthalate (BDH)

Methanol

Crystal-violet Indicator

Phenol red

Ammonia (BDH)

Mercuric Acetate (ACS)

Table 2: Profiles of pure samples used

Name	Batch no	Man. Date	Expiry Date	Assay%
Ciprofloxacin	101119-2	11/2010	11/2013	99.4
Salicylic acid	300384B	–	–	99.5
Benzoic acid	1856	–	–	99.5

Pure Ciprofloxacin hydrochloride was obtained from Ernest Chemist Ltd where as Salicylic acid and Benzoic acid were provided by the Department of Pharmaceutical Chemistry, KNUST, Kumasi.

Five brands of Ciprofloxacin tablets and four brands of Ciprofloxacin intravenous infusion from different manufacturing companies were purchased from retail shops around Kumasi metropolis. Their descriptions are as follows:

Table 3: Profile of Ciprofloxacin tablets sample

Tablet Name	Tablet strength(mg)	Name of manufacturer	Batch no.	Manufacturing date	Expiry date
UC	500	Unichem Industries Ltd, Ghana	11	05/2012	04/2014
MC	500	Mission Vivacare Ltd, India	PC2111001A	04/2011	03/2014
DC	500	Danadams Pharmaceuticals Ltd, Ghana	12050905	05/2012	05/2015
SC	500	Sravani Laboratories Ltd, India	CP-01	04/2011	03/2014
EC	500	Ernest chemist Ltd. Ghana	1011L	11/2011	11/2015

Table 4 Profile of Ciprofloxacin infusions sample

Infusion name	Infusion strength(mg)	Name of manufacturer	Batch no.	Manufacturing date	Expiry date
ECI	200	Ernest Chemist Ltd, Ghana.	C-10/11/101	06/2011	05/2014
MCI	200	Marck Bioscience Ltd, India	22110648	12/2011	11/2014
SCI	200	Shalina Lab. PVT Ltd, India	82EK423009	10/2011	09/2014
LCI	200	Luex pharmaceutical, UK	82EF423001	06/2011	05/2014

3.0.2 Instrumentation

- Shimadzu LC-20AB Prominence Liquid Chromatograph Pump
- Kontron Instrument HPLC Pump 422
- eDAQ Power Chrom Software 280
- Perkin Elmer 785 UV/VIS Detector
- HP Computer work station
- Bondclone 10 Phenomenex C18 300 x 3.90mm
- HP Computer Workstation
- Adam PW 124 weighing balance
- Eutech Instrument pH 510/ pHmV/ ^{o}C meter
- Fischer Scientific FS 28H sonicator
- Stuart melting point SMP 10 apparatus
- Whatman filter paper
- Chromato-Vue C-70G UV viewing system (UVP inc)
- Melting point capillary tube

3.0.3 Identification tests for pure samples

3.0.3.1. Qualitative test

3.0.3.1.1 Ciprofloxacin hydrochloride pure sample

1. Ciprofloxacin hydrochloride was weighed to 0.5g, dissolved in carbon dioxide free water diluted to 20ml. The colour of the solution was then observed and the pH taken.

2. 0.001g of Ciprofloxacin was weighed and dissolved with water into a 10mL volumetric flask and shaken. A quantity of the solution was poured into a cuvette and it was scanned within a wavelength range of 225 to 350nm using a T-90+ UV/ VIS Spectrophotometer.

3.0.3.1.2 Benzoic acid pure sample

0.5g of benzoic acid was dissolved in a conical flask with ethanol and diluted to10ml with the same solution. After observation of its colour 1ml of the solution was taken and 0.5ml of ferric chloride solution added. The colour was then observed again after the addition of ether.

3.0.3.1.3 Salicylic acid pure sample

30mg of salicylic acid pure powder was weighed, dissolved in 5ml of 0.05M sodium hydroxide and diluted to 20ml with water. To1ml of this solution 0.5ml ferric chloride was added and followed with 0.1ml acetic acid. The solution was then observed.

3.0.3.2 Melting point determination

Separate capillary tubes sealed at one end were filled with dry powders of each pure sample. The tubes were gently tapped on the sealed end to obtain well packed column, about 3mm in height. The filled ends were placed in melting point determination equipment and their respective melting points noted.

3.0.4 Assay of pure samples

3.0.4.1 Ciprofloxacin

Ciprofloxacin hydrochloride pure powder, 0.2g, was weighed and dissolved in 15ml glacial acetic acid, followed by the addition of 1ml freshly prepared mercuric acetate solution and 4ml of acetic anhydride. The solution was then titrated against 0.1M acetous perchloric acid using

crystal violet as indicator until a yellowish green end point. Blank titration was carried out and the titre values calculated. The procedure was carried out in triplicate.

3.0.4.2 Salicylic acid

To 0.120g of salicylic acid, ethanol was added and diluted to 30ml. 20ml of water was added to the solution and titrated with 0.1M sodium hydroxide, using phenol red as indicator. 1ml of 0.1M sodium hydroxide is equivalent to 13.81mg of $C_7H_60_3$

3.0.4.3 Benzoic acid

0.2g of benzoic acid was weighed and dissolved in 20ml of ethanol. The solution was titrated with 0.1M sodium hydroxide using phenol red as indicator, until a colour change from yellow to violet. 1ml of 0.1M sodium hydroxide is equivalent to 12.21mg of $C_7H_6O_2$.

3.0.5 Standardization of solutions

3.0.5.1 Standardization of 0.1M perchloric acid

0.5005g of Potassium hydrogen Phthalate was weighed to into a conical flask and dissolved with 25ml glacial acetic acid. The solution was warmed to ensure complete dissolution of the salt. After cooling the solution was titrated with 0.1M perchloric acid, using oracet blue as indicator.

3.0.5.2 Standardization of sodium hydroxide

25ml of sulphamic acid solution was pippetted into a conical flask and titrated against 0.1M sodium hydroxide using methyl orange indicator. A triplicate determination was then made.

3.0.6 Thin Layer chromatography (TLC) of drug samples

3.0.6.1 Ciprofloxacin tablets

Test solutions of the various brands of ciprofloxacin tablets were prepared by dissolving a quantity of the powdered tablets containing 50mg of ciprofloxacin hydrochloride in water. The solution was diluted to 100ml with water and filtered. Reference solution from the pure powder was also prepared to a concentration of 0.05% in the same solvent.

Using silica gel coated plate and a mobile phase composition of 10 volumes of acetonitrile, 20 volumes of ammonia, 40 volumes of methanol, and 40 volumes dichloromethane TLC was carried out. 10µL of each brand was spotted separately with the reference solution on a plate and allowed to dry. The plates were placed in a chamber containing the mobile phase to develop.

The plates were removed dried and observed under UV light. The Rf values of both the sample and standard were then compared.

3.0.6.2 Ciprofloxacin infusion

Sample solutions were prepared by pipetting a quantity of the intravenous infusion and diluting it to 0.05% with water, for each brand. A standard solution was also prepared by dissolving 50mg of the pure powder in water and further diluted to 0.05% with the same solvent.

Chromatography was carried out using TLC plate coated with a 0.2mm layer silica gel as the stationary phase and a mobile phase of acetonitrile, ammonia, methanol and dichloromethane. (1:2:4:4)

10µL portions of the sample solution and the standard solution each were spotted on a TLC plate, allowed to dry and the plate developed in a chamber containing the mobile phase. After development the chromatogram was then observed under UV light and the Rf values determined.

3.0.7 Assay of Ciprofloxacin tablets and infusions

3.0.7.1 Uniformity of weight of Ciprofloxacin Tablets

For each of the five brands of ciprofloxacin tablets 20 tablets were weighed individually and recorded. The average weight of the tablet was determined for each of the brands. Then the deviation and percentage deviations were calculated for each of the weights taken.

3.0.7.2 Determination of the Percentage content of Ciprofloxacin in Ciprofloxacin tablets using a Standard method

20 tablets of a Ciprofloxacin brand were weighed accurately and powdered. A quantity of the powdered sample equivalent to 200mg was weighed, dissolved with 15ml of glacial acetic acid using a sonicator and filtered using whatman filter paper. This was followed by the addition of 1ml freshly prepared mercuric (II) acetate and 4ml acetic anhydride. The solution was then titrated against 0.1M perchloric acid using crystal violet indicator until a yellowish-green colour endpoint. Blank titration was carried out and adjustment made to get the titre value. The procedure was carried out in a triplicate and applied to the four other brands of Ciprofloxacin tablets.

3.0.7.3 Determination of the Percentage content of Ciprofloxacin in Ciprofloxacin infusion

0.5ml of the intravenous infusion was diluted to make 0.01% w/v with sufficient amount of the mobile phase to make up the sample solution. A reference solution was prepared by dissolving 10mg of the pure powder with the mobile phase to 0.01% w/v with the aid of a sonicator. 5µl of

each solution was injected separately. The chromatographic procedure involved a stainless steel C-18 column (300 x 3.9mm), a mobile phase mixture of 50 volumes of methanol, 45 volumes of water and 5 volumes of phosphate buffer (pH- 3) solution, at a flow rate of 1.5mL per minute at a wavelength of 278nm. The content of ciprofloxacin was then calculated. Each mg of Ciprofloxacin hydrochloride is equivalent to 0.9010mg of ciprofloxacin.

3.0.8 HPLC Method Development

3.0.8.1 Chromatographic system for Ciprofloxacin and its surrogates

In the method development, mixture of methanol, water and KH_2PO_4, at different pH's, in different combinations were investigated at different flow rates to determine the effect on elution of ciprofloxacin as well as the surrogate reference standards. The condition suitable was methanol: water: phosphate buffer (pH-3) in the ratio 50:45:5, as mobile phase at a flow rate of 1.5mL/min for better resolution. C-18 Phenomenex 300 x 3.9mm column reverse phase was chosen as the stationary phase.

On the basis of the UV absorption maximum of the drug samples used over the range of 225nm to 350nm, 239nm was the wavelength chosen for monitoring to provide the appropriate intensity for all the target compounds.

3.0.8.2 Preparation of Mobile phase

A mobile phase combination of methanol, water and phosphate buffer (10:9:1) was able to elute Ciprofloxacin and its chosen surrogates. The buffer solution was prepared by dissolving 0.34g of potassium dihydrogen orthophosphate in 90ml of water, adjusting the pH to 3.0 with phosphoric acid and diluting the solution to100ml with water. The solution was then filtered.

3.0.8 3 Determination of the wavelength of Maximum Absorption

Ciprofloxacin pure powder was weighed to 10mg and poured into a 100mL volumetric flask. The mobile phase was added, shaken vigorously for complete dissolution and diluted with the same solvent to make up to volume. 5µl of the solution was injected into the chromatograph and a UV scan carried out, using the Shimadzu LC-20AB prominence liquid chromatograph.

3.0.8.4 Stability Study of the Drugs Used

In order to ascertain the stability of the drug samples that were employed, in solution, within the time frame of the experiment a stability study was conducted. The various drugs were prepared at a known concentration. The sample solutions were injected six times at an interval of 10 minutes for one hour and the peak areas noted. A graph of peak area against time was then plotted.

3.0.9 Analytical Performance Parameters

3.0.9.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

From a stock concentration of 0.01% w/v for all the samples five other concentrations were prepared. 100µl of each of these resultant concentrations were injected into the column one after the other. The peak areas were measured from the chromatograms. The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were then determined using the formula below;

$LOD = 3.3\sigma / S$ and $LOQ = 10\sigma / S$ [79]

Where; σ = residual standard deviation ie. $\sigma_{res} = \{\Sigma(Y - Y_{est})^2 / n-1\}$,

Where: Y = y values (Area) from a calibration curve and Y_{est} = y values calculated using the equation of line $y = mx + c$

S = the slope of the equation of line from the calibration curve drawn.

3.0.9.2 Linearity

Stock solutions of the analyte and the surrogate reference standards were prepared to 0.01%w/v and serially diluted to different concentrations. 100µl of these various concentrations of solutions were injected into the column. The peak areas were noted and plotted against the concentrations on a graph.

3.0.9.3 Repeatability and Intermediate Precision

The analyte and surrogates were prepared at various concentrations and injected five times in a day on three different occasions. For each concentration of the analyte and surrogates the injections were repeated to test for the precision, within and in-between run.

3.0.9.4 Accuracy and Precision

After the acquisition of the various peak areas and percentage contents based on the standard method and the developed method, tests were carried out to ascertain the accuracy and precision of the derived procedure.

3.0.10 Determination of K Using the Surrogate Reference Standards

The analyte and the surrogate reference standards were prepared to 0.01%w/v and from this other concentrations were prepared by serial dilutions. The analyte and a surrogate were mixed in equal amounts on each occasion and injected into the column. On each occasion the chromatograms were recorded and the peak areas measured. From this the constant K for each surrogate reference standard against the analyte was calculated with the formula provided in the hypothesis in chapter one.

3.0.11 Analysis of Ciprofloxacin tablets and infusion using the surrogate Reference Standards

3.0.11.1 Ciprofloxacin tablets

Twenty tablets of each of the five different brands of Ciprofloxacin tablets were powdered. An amount of the powder containing 10mg pure Ciprofloxacin was dissolved in 10mL of the mobile phase and sonicated. A portion of the resulting solution was filtered using a micro filter. 1mL of the resulting solution was dissolved in 10mL of the same solvent and subsequently to 0.005% w/v. For the surrogate reference standards 0.005%w/v was also prepared by dissolution in methanol and filtered using a micro filter. 5mL each of the analyte and the surrogate reference standard were mixed and 100µL of the resulting solution injected. The peak areas were recorded from the chromatograph and calculations done to obtain the percentage content. This was done for the analyte against all the two surrogates.

3.0.11.2 Ciprofloxacin infusion

0.5ml of the infusion was pippetted and dissolved with the mobile phase to 0.01%w/v. The surrogates were then prepared with methanol to 0.01%w/v by weighing 10mg followed with dissolution in a volumetric flask. These solutions were filtered with a micro filter and mixed by the addition of 5ml of each solution. 100µL of the resultant solution was injected into the chromatogram and the major peak areas identified. This was repeated for the other surrogate.

CHAPTER FOUR

4.0 RESULTS AND CALCULATION

4.1 Identification of Pure samples

4.1.1 Qualitative test

4.1.1.1 Colour and U. V test

Table 5: Results of tests

Sample	Result	Inference
Ciprofloxacin	1. The solution was clear with a pH of 3.9. 2. The solution showed an absorption maxima at 274 and minimum at 320	Positive [9] Positive[45]
Benzoic acid	1. The solution was clear and colourless 2. A yellow precipitate soluble in ether was formed	Positive [9] Positive [9]
Salicylic acid	1. The solution produced a violet colour that persisted after the addition of acetic acid.	Positive [9]

4.1.1.2 Melting point of the samples

Table 6: Melting points determined

Sample	Literature range(^{O}C)	Observed range(^{O}C)
Ciprofloxacin	318 – 320	_
Benzoic acid	121 – 124	123-125
Salicylic acid	158 – 161	157-158

4.2 Standardization of solutions

4.2.1 Standardisation of 0.1M NaOH with Sulphamic acid

For sulphamic acid 99% = 0.9709g

: 100% = (100/99) x0.9709g

= 0.9807g

Factor of H_2NSO_3H = Actual weight/ nominal weight

=0.9866/0.9807

F(H_2NSO_3H) =1.0060

Factor(H_2NSO_3H) x Volume(H_2NSO_3H) = Factor(NaOH) x Volume(NaOH)

F(NaOH) = [F(H_2NSO_3H) x V(H_2NSO_3H)] / V(NaOH)

F(NaOH) = (1.0060 x25ml) / 26.15

F(NaOH) = 0.9618

4.2.2 Standardization of 0.1MPerchloric acid with Potassium Hydrogen Phthalate

204.14g of $KC_8H_5O_4$ in 1000ml ≡ 1M $HClO_4$

2.0414g of $KC_8H_5O_4$ in 100ml ≡ 0.1M $HClO_4$

Factor of $KC_8H_5O_4$ = Actual weight/nominal weight

=0.5004/0.5000

= 1.0008

Similar calculations of two other determinations gave 1.0080 and 1.0054

Average factor =1.0054+1.0080+1.0008 =1.0047

3

Mole ratio between $KC_8H_5O_4$ and $HClO_4$ is 1:1

Average titre= 25.80

F ($HClO_4$ =[F($KC_8H_5O_4$) x V($KC_8H_5O_4$)] /V($HClO_4$)

= 1.0047x25.00

25.80

F ($HClO_4$) = 0.9735

4.3 Assay of Analyte and Surrogates

4.3.1 Benzoic acid

Factor of NaOH is 0.9618

Actual volume of NaOH = titre x F(NaOH)

=17.1 x 0.9618

=16.4468ml

1ml of 0.1 M sodium hydroxide is equivalent to 12.21mg of $C_7H_6O_2$

Therefore Actual amount =16.4468 x 0.01221

=0.2008g

Percentage purity = Actual x 100

Nominal

= (0.2008/0.2013) x 100%

=99.76%

A second determination yielded 98.81%

Average %purity = (99.76 + 98.81)/2

=99.3%

4.3.2 Salicylic acid

F(NaOH) = 0.9618

Actual volume of NaOH for the determination = titre x F(NaOH)

=8.9 x 0.9618

=8.56ml

From the milliequivalent

1ml of 0.1M sodium hydroxide is equivalent to 13.81mg of $C_7H_6O_3$

Actual amount = 8.56 x 0.01381

= 0.1182g

%purity = (actual wt/ nominal wt) x100%

= (0.1182 /0.1198) x100

=98.7%

A second determination gave 99.3%

Average %purity = $\frac{99.3+98.7}{2}$

= 99.0%

4.3.3 Ciprofloxacin

F ($HClO_4$) = 0.9735

Titre =6.2 – 0.4 (blank) = 5.8ml

Actual titre = 5.8 x 0.9735

= 5.6463ml

From milliequivalent deductions

1ml of 0.1M perchloric acid is equivalent to 0.03678g of $C_{17}H_{18}FN_3O_3$, HCl

Actual amount =5.6463 x 0.03678 = 0.2077g

%purity of ciprofloxacin hydrochloride = (0.2077/0.2071) x100

=100.3%

Another determination yielded 97.5%

Averagely the %percentage purity is 98.9%

4.4 Thin Layer Chromatography (TLC)

4.4.1 TLC for Ciprofloxacin Tablets

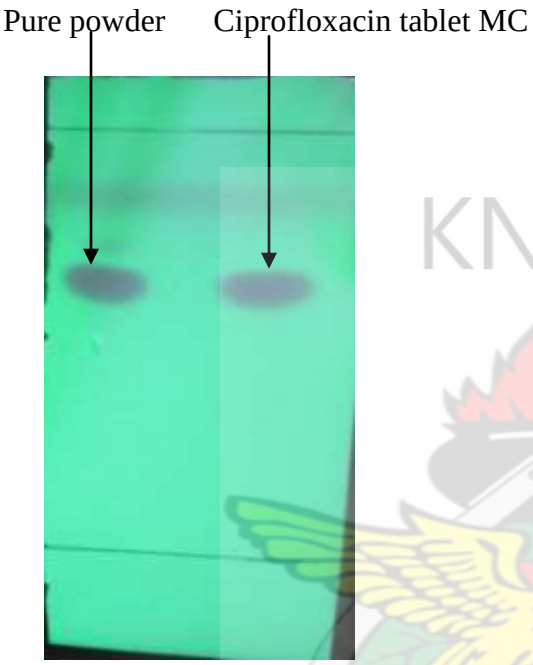

Figure 6: TLC for a Ciprofloxacin tablet, Unichem Ind. Ltd and the Reference standard

Rf Value = Distance travelled by component from the origin
Distance travelled by mobile phase front from the origin

In the case of Ciprofloxacin tablet UC from Unichem Pharmaceuticals Limited, Ghana the Rf was found as the following

Rf = 3.2/4.6

=0.70

4.4.2 TLC for Ciprofloxacin infusions

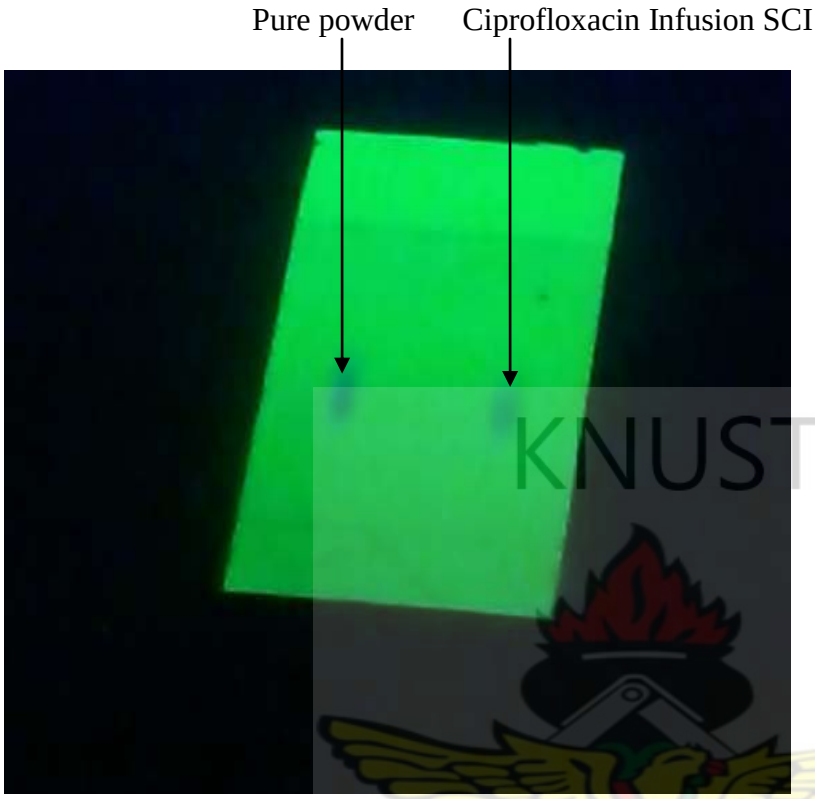

Figure 7: TLC for Ciprofloxacin infusion from Marck Biosciences Ltd. and the Reference standard

All the Rf values for the tablets and infusions of various brands subsequently gave the following Rf values:

Table 7: Results of Rf values for the various brands of Ciprofloxacin tablets

Brand name	Rf value for pure sample	Rf value for tablet
UC	0.696	0.696
MC	0.700	0.700
DC	0.680	0.680
SC	0.684	0.684
EC	0..690	0.690

Table 8: Results of Rf values for the various brands of Ciprofloxacin infusion

Brand name	Rf value for pure sample	Rf value for tablet
ECI	0.700	0.700
MCI	0.682	0.682
SCI	0.675	0.675
LCI	0.700	0.700

4.5 Uniformity of weight

Refer to table U1 to U5 in the appendix

4.6 Assay of Ciprofloxacin tablets and infusions

4.6.1 Determination of percentage content of Ciprofloxacin in Ciprofloxacin tablet (500mg)

A sample calculation with UC brand of Ciprofloxacin tablet yielded this result

F ($HClO_4$) = 0.9735

Titre =6.1 – 0.4 (blank) = 5.7ml

Actual titre = 5.7 x 0.9735(factor of perchloric acid)

= 5.5490ml

From milliequivalent deductions

1ml of 0.1M perchloric acid is equivalent to 0.03678g of $C_{17}H_{18}FN_3O_3$, HCl

Actual amount =5.5490 x 0.03678 = 0.2041g

From the uniformity of weight 0.9483g of the powdered sample contains 0.5g of ciprofloxacin

Therefore 0.3793g contains 0.2g of the active ingredient, but 0.3826g was weighed, containing 0.2017g of Ciprofloxacin.

%purity of ciprofloxacin = (0.2041/0.2017) x100

=101.19%

Table 9: Table of results for the percentage content of various brands of Ciprofloxacin tablet

Brand name	Percentage Content					
	1	2	3	4	5	Mean Percentage content
UC	101.19	100.8	99.92	101.36	99.85	100.62 ±0.32
MC	96.85	97.72	95.34	94.72	97.69	96.47 ±0.61
DC	94.25	95.93	96.67	95.32	96.97	95.83 ±0.49
SC	99.24	98.88	99.54	100.23	99.02	99.38 ±0.24
EC	97.14	97.65	96.54	98.26	96.58	97.23 ±0.33

4.6.2 Determination of percentage content of Ciprofloxacin in Ciprofloxacin infusion

Like all the other brands of infusions calculation of the percentage content for MCI was computed with this relation:

$$\frac{Aa}{Ca} = \frac{As}{Cs}$$

Where, Aa is the peak area of the Ciprofloxacin tablets

As is the peak area of the Ciprofloxacin pure sample

Cs is the concentration of the Ciprofloxacin pure sample

Ca is the concentration of the Ciprofloxacin tablets

Thus, $Ca = \frac{Aa \times Cs}{As}$

However, each mg of $C_{17}H_{18}FN_3O_3$,HCl is equivalent to 0.9010 mg of $C_{17}H_{18}FN_3O_3$. Hence 0.01%w/v is equivalent to $C_{17}H_{18}FN_3O_3$ at 0.00901%w/v.

Aa = 2.27

As = 2.56

Cs = 0.01%w/v

$$= \frac{2.27 \times 0.01}{2.56}$$

$$= 0.00887\%w/v$$

The Percentage content = (Actual concentration / nominal concentration) x 100

$$= \frac{0.00887}{0.00901} \times 100$$

$$= 98.45\%w/v$$

Table 10: Results obtained for the assay of Ciprofloxacin infusions

Brand name	**Batch number**	**Percentage Content**					
		1	**2**	**3**	**4**	**5**	**Mean Percentage content**
ECI	C-10/11/101	96.28	96.6	95.32	97.64	95.58	96.28 ± 0.41
MCI	22110648	98.45	100.12	99.68	101.22	100.42	99.98 ± 0.46
SCI	82EK423009	95.26	95.64	97.65	98.68	97.21	96.89 ± 0.64
LCI	82EF423001	98.34	98.45	97.56	99.12	97.82	98.26 ± 0.27

Table 4.7 HPLC Development

Table 11: Mean retention times for ciprofloxacin and the surrogates used

Sample	Mean retention time
Ciprofloxacin	2.68 ± 0.20
Salicylic acid	4.44 ± 0.15
Benzoic acid	4.69 ±0.20

4.7.2 Chromatograms for the Ciprofloxacin tablets, infusions and surrogates used

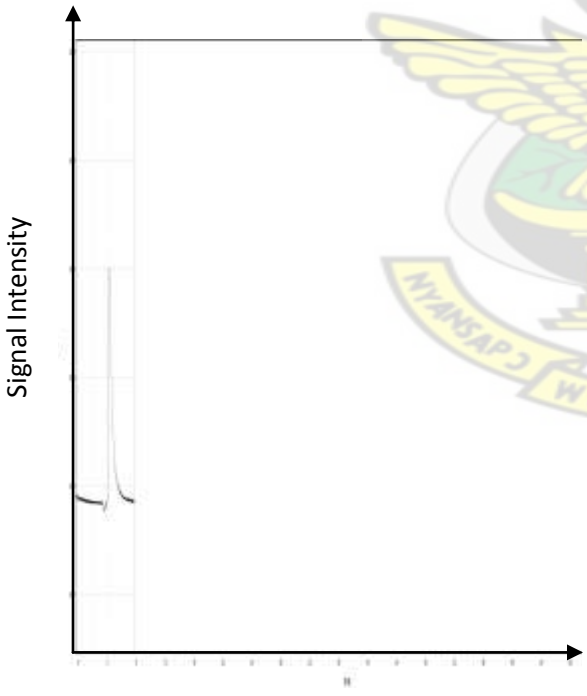

Figure 8: chromatogram of pure ciprofloxacin

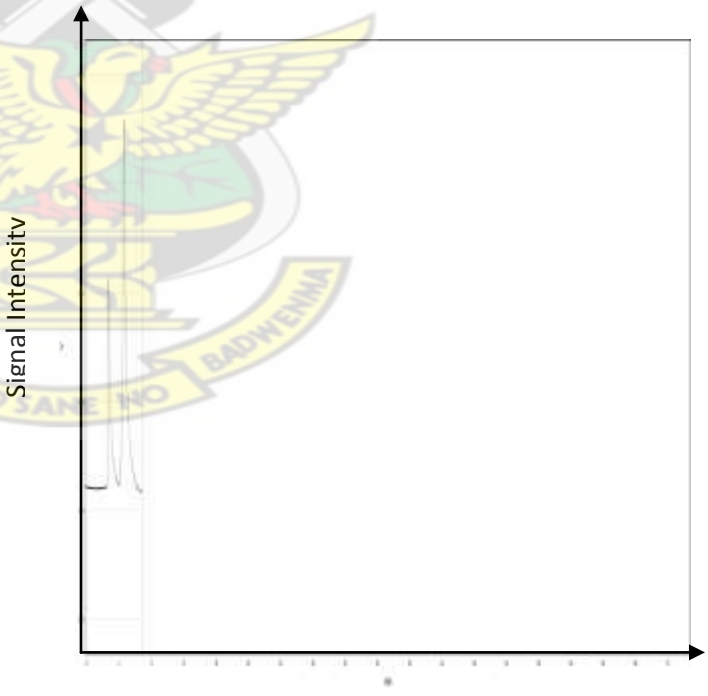

Figure 9: chromatogram of UC and Salicylic acid

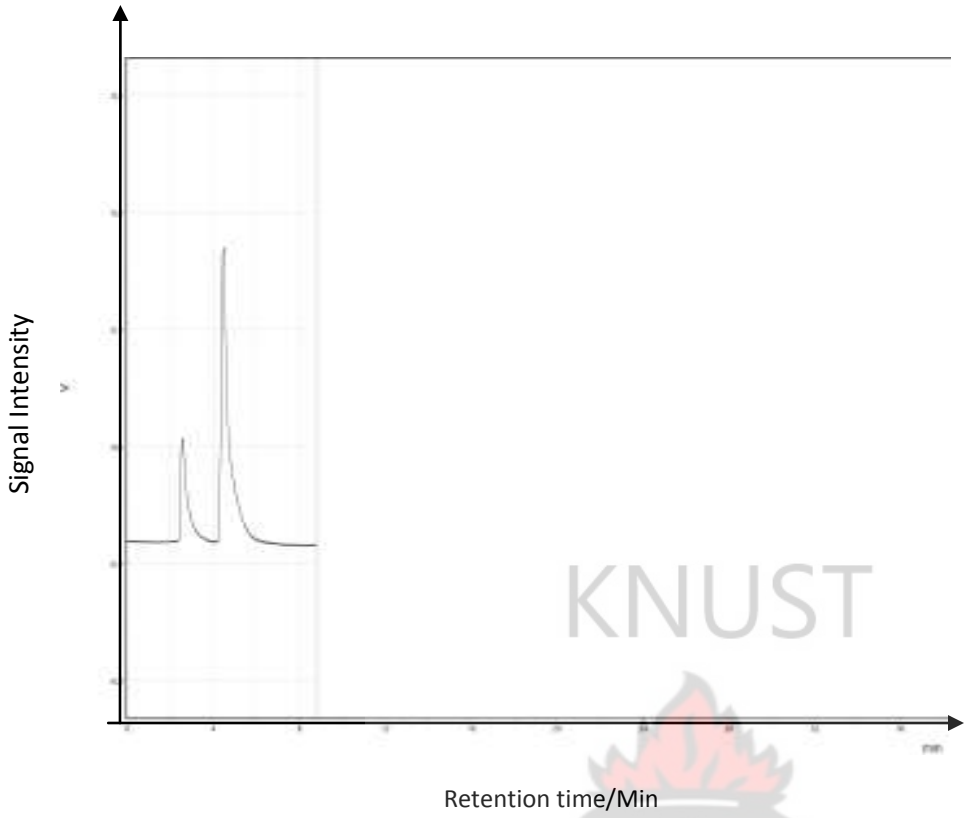

Figure 10: Chromatogram of MC and Salicylic acid as surrogate

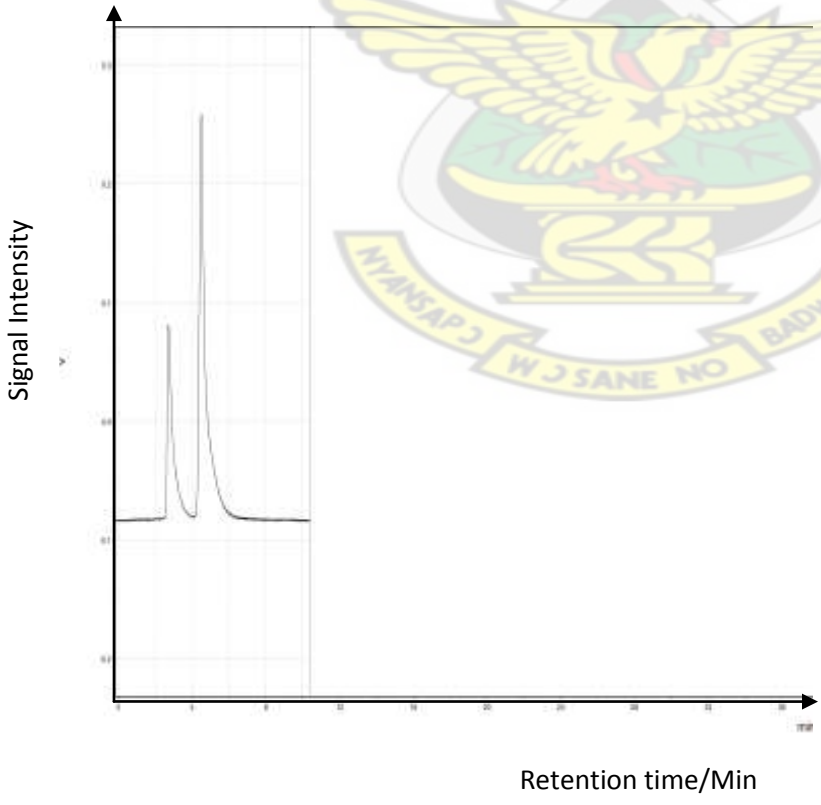

Figure 11: Chromatogram of DC and Salicylic acid as surrogate

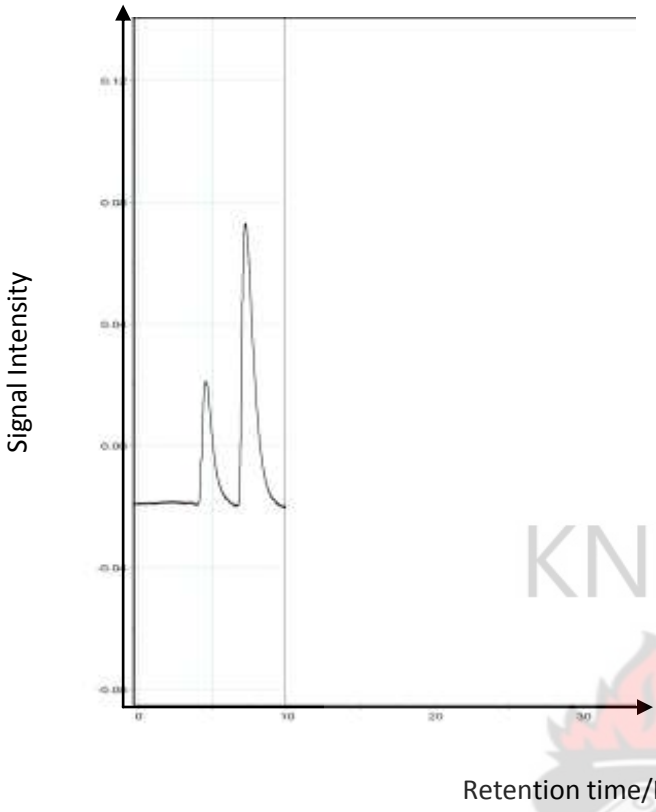

Figure 12: Chromatogram of Ciprofloxacin tablet, DC and Benzoic acid as surrogate

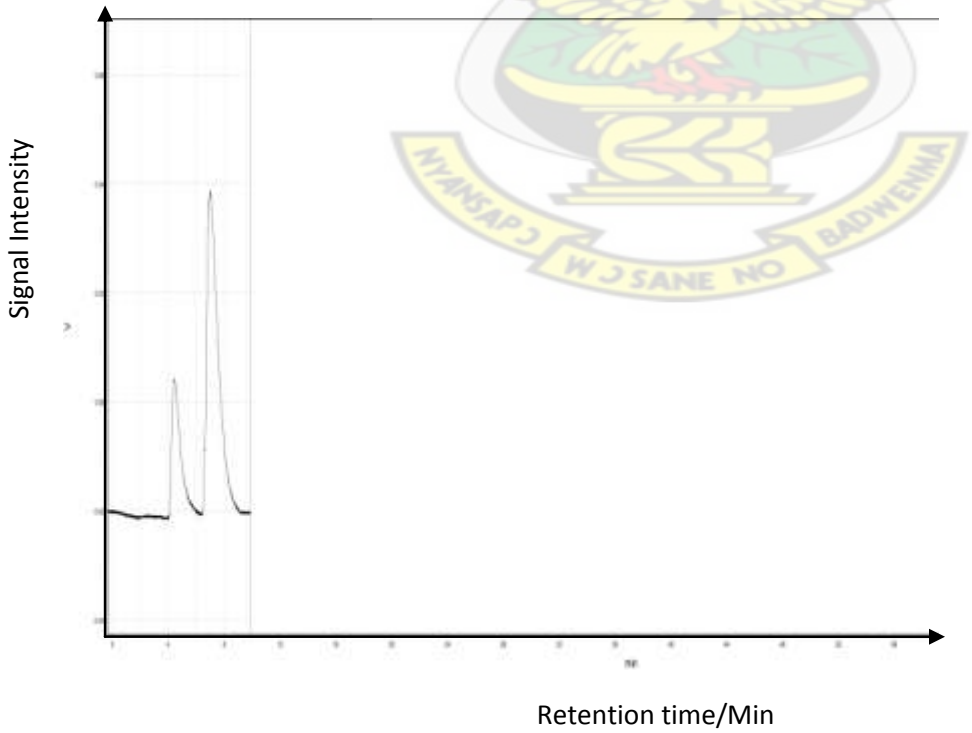

Figure 13: Chromatogram of Ciprofloxacin tablet, EC and Benzoic acid as surrogate

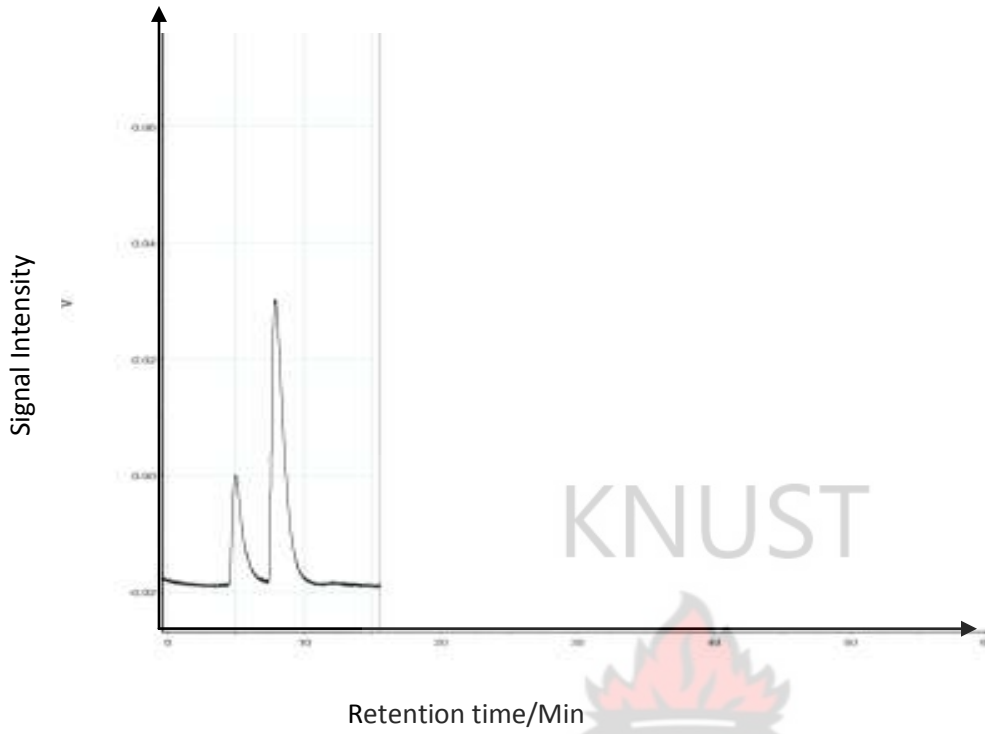

Figure 14: Chromatogram of Ciprofloxacin tablet, SC and Benzoic acid as surrogate

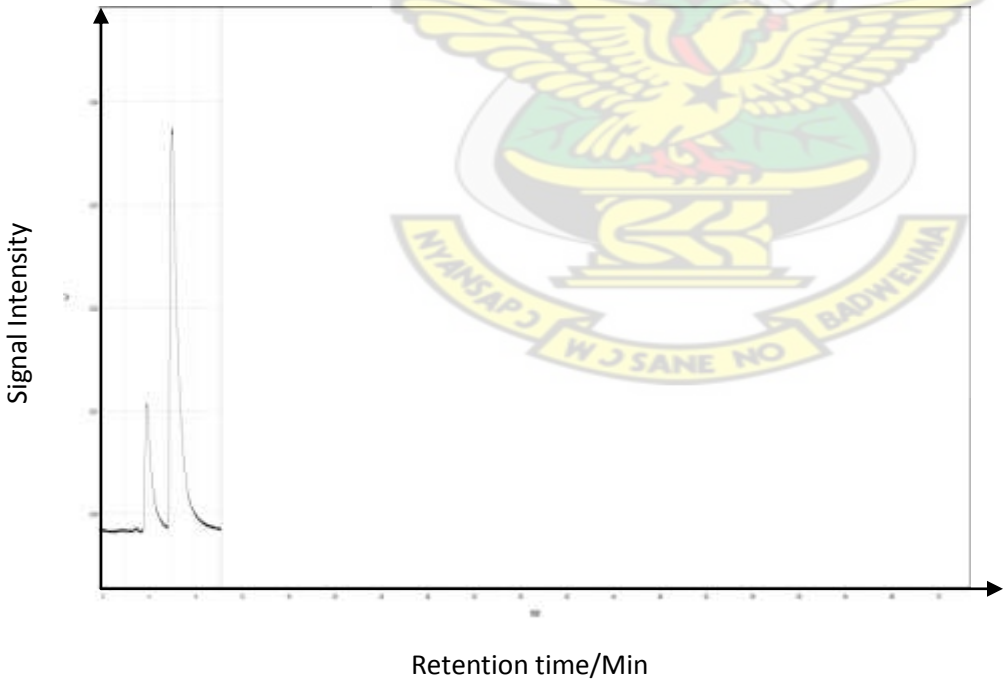

Figure 15: Chromatogram of Ciprofloxacin infusion, ECI and Salicylic acid

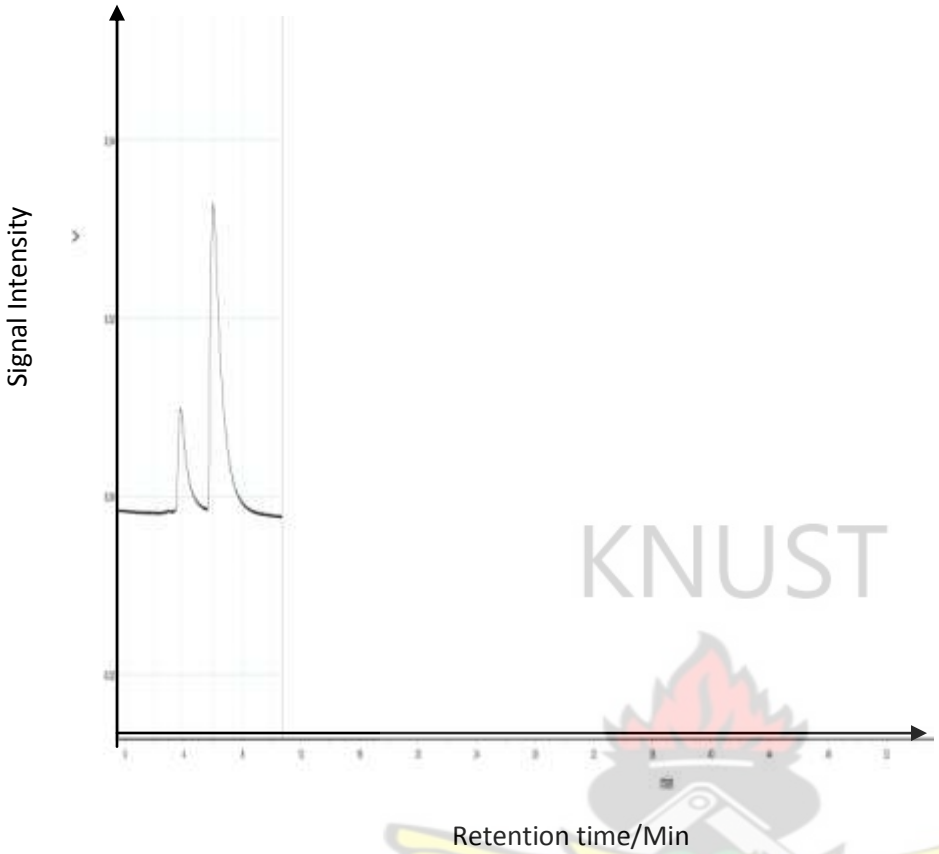

Figure 16: Chromatogram of Ciprofloxacin infusion, MCI and Salicylic acid as surrogate

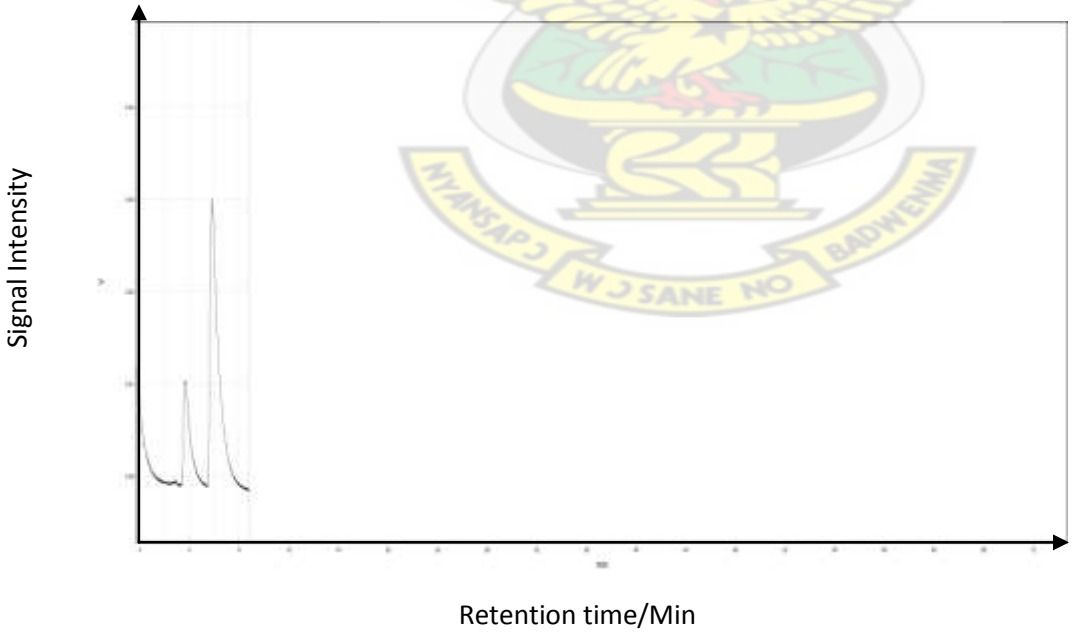

Figure 17: Chromatogram of Ciprofloxacin infusion LCI and Salicylic acid as surrogate

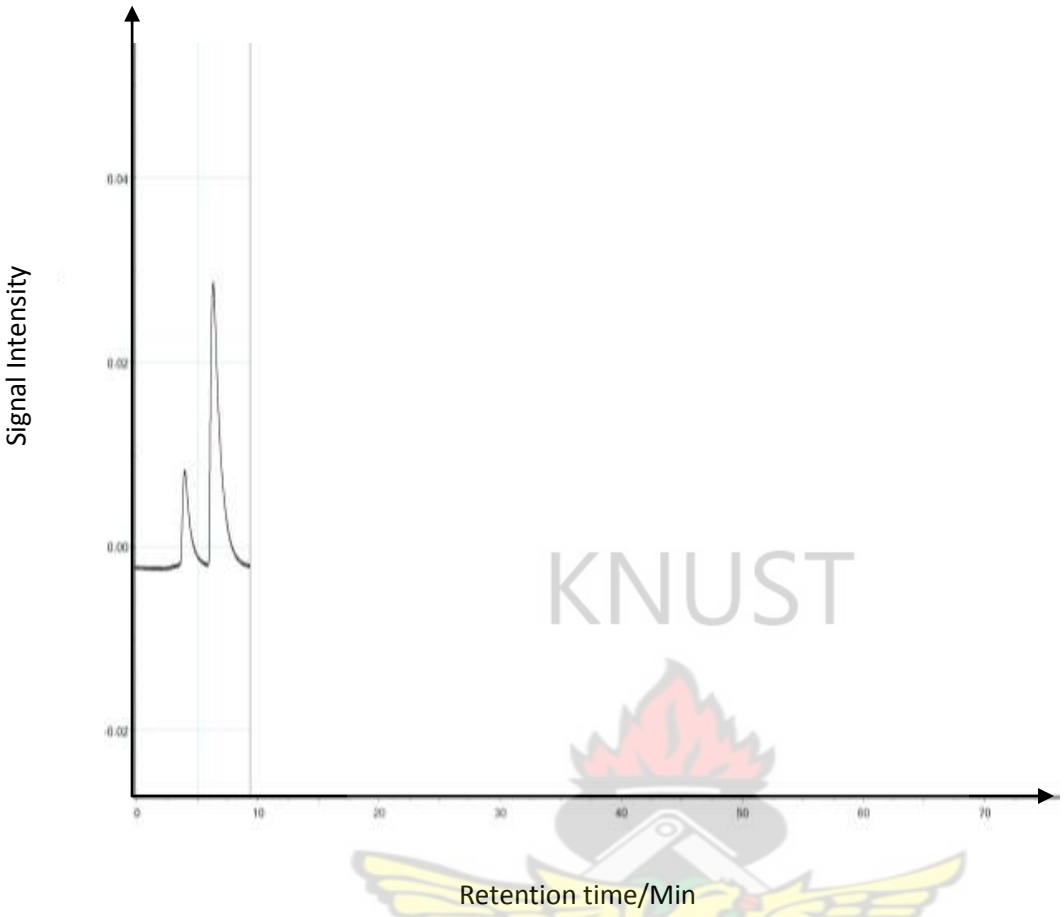

Figure 18: Chromatogram of Ciprofloxacin infusion SCI and Salicylic acid

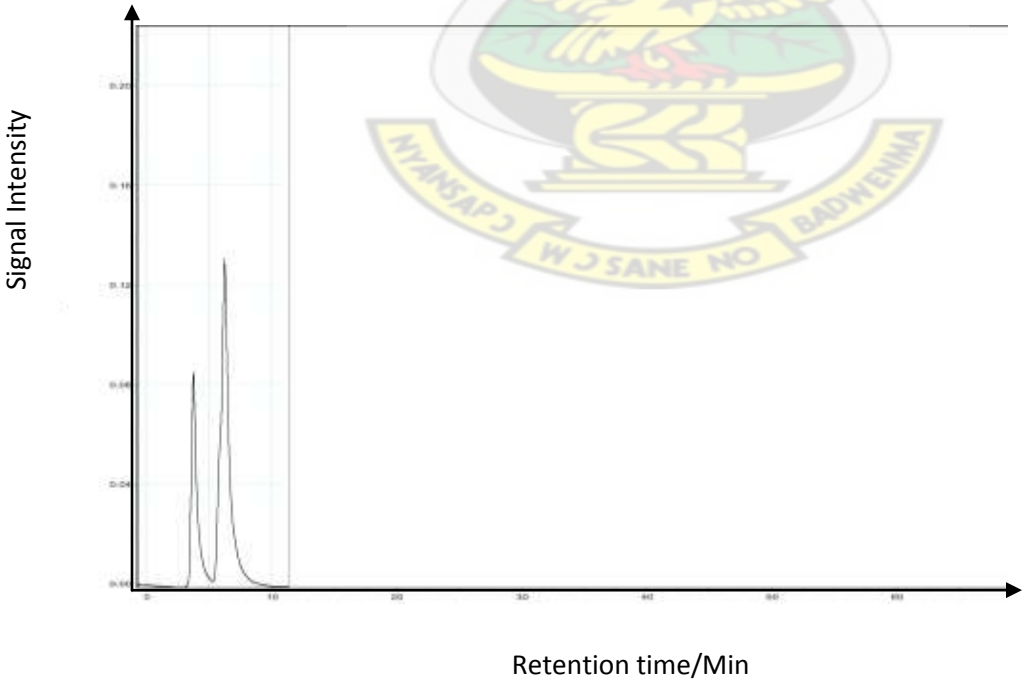

Figure 19: Chromatogram of Ciprofloxacin infusion ECI and pure Benzoic acid

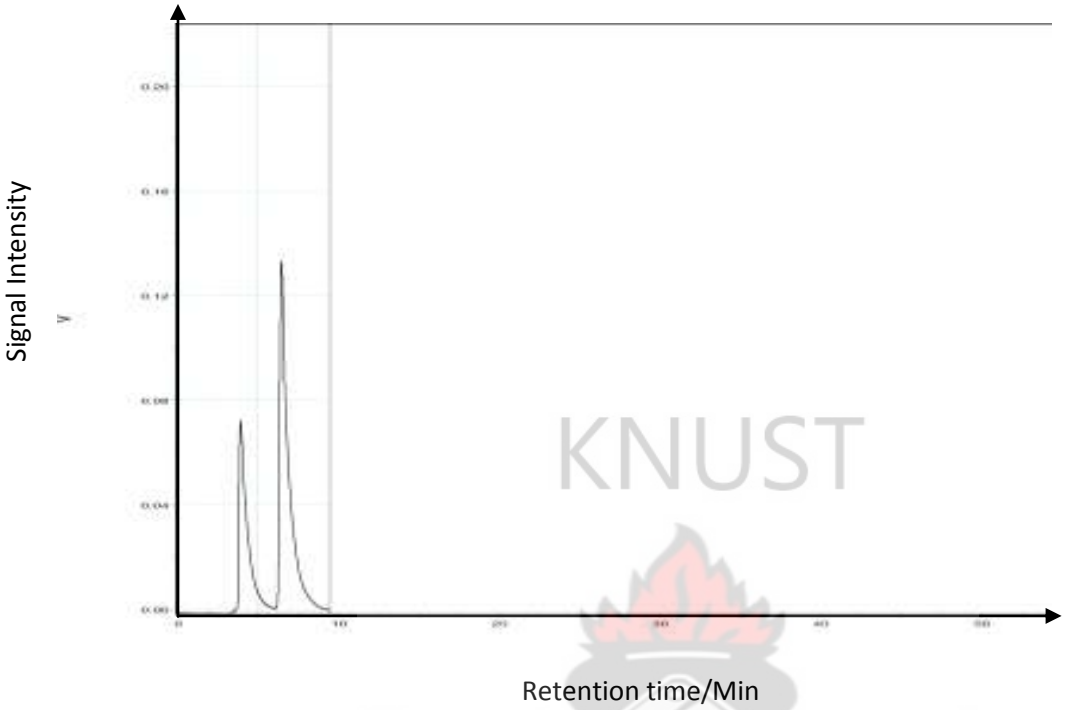

Figure 20: Chromatogram of Ciprofloxacin infusion MCI and pure Benzoic acid

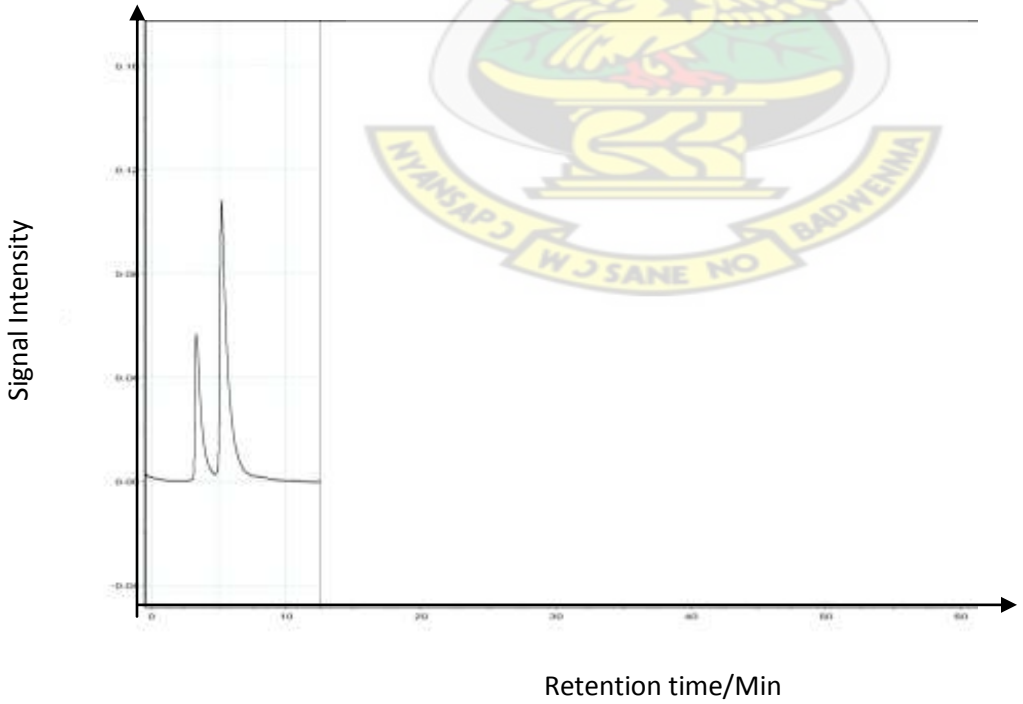

Figure 21: Chromatogram of Ciprofloxacin infusion LCI and Benzoic acid

4.7.3 Calibration curves for Ciprofloxacin and the Surrogates

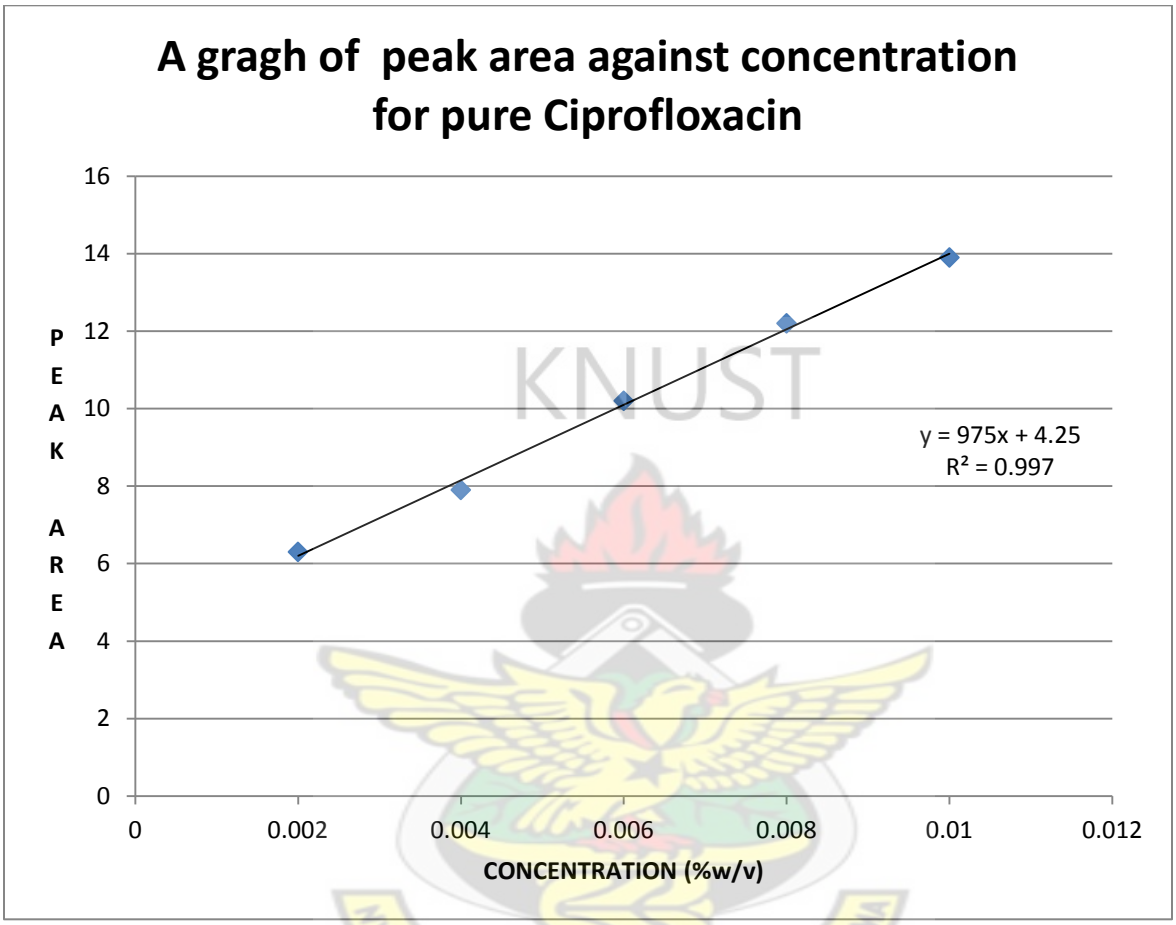

Figure 22: Calibration curve for Pure Ciprofloxacin

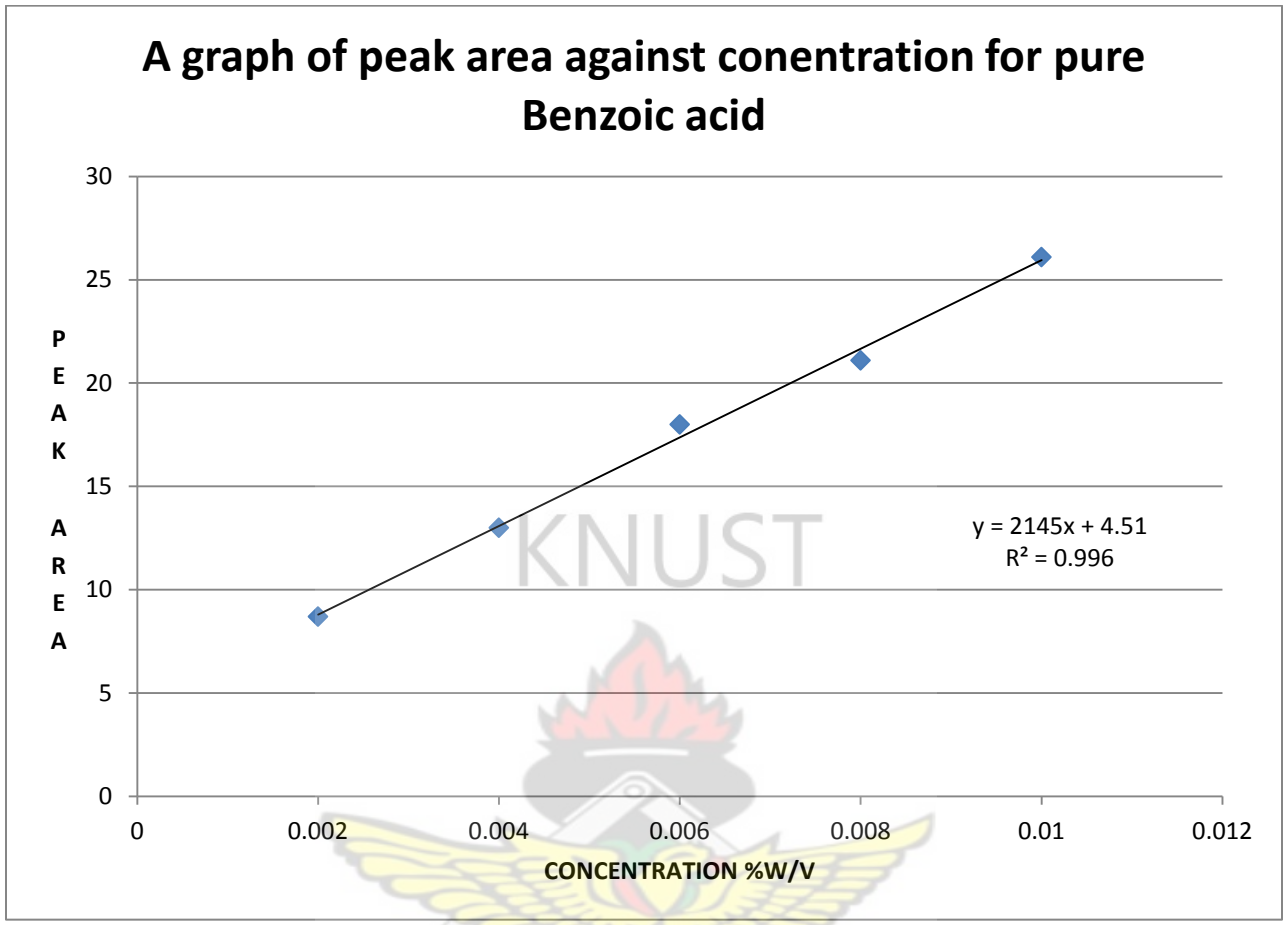

Figure 23: Calibration Curve for Pure Benzoic acid

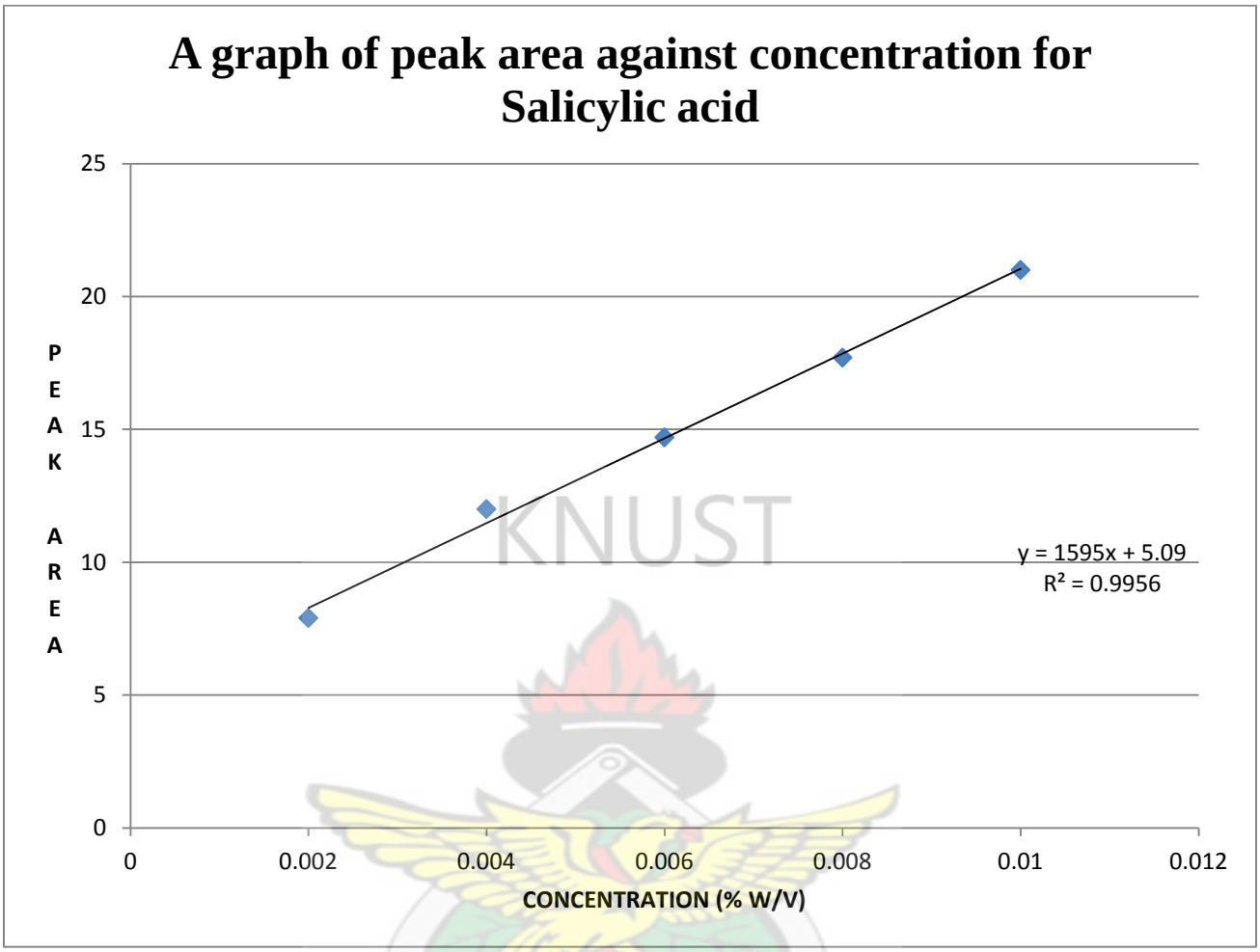

Figure 24: Calibration curve for salicylic acid

4.7.4 Analytical Performance Parameters

4.7.4.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD is given by LOD = 3.3σ / S and LOQ = 10σ / S,

σ is the residual standard deviation obtained by $\sigma_{res} = \{\Sigma(Y - Y_{est})^2 / n-1\}$,

Y is the y value from the calibration curve and Y_{est} is the y calculated from the equation of the line, y = mx + c

S is the gradient of the line

Table 12: Determination of the LOD and LOQ for pure Ciprofloxacin

Concentration X	**Peak area Y**	$\mathbf{Y_{est}}$	$\mathbf{Y-Y_{est}}$	$\mathbf{(Y-Y_{est})^2}$
0.01	13.9	14	-0.1	0.01
0.008	12.5	12.05	0.15	0.0225
0.006	10.20	10.1	0.1	0.01
0.004	7.90	8.15	-0.25	0.0625
0.002	6.3	6.2	0.1	0.01

$\sum(Y - Y_{est})^2 = 0.115$

Number of degrees of freedom = n -1= 4

$\sigma_{res} = \sum(Y - Y_{est})^2 / n-1$

$= 0.115/4$

$\sigma_{res} = 0.02875$

$LOD = 3.3\sigma / S$

$= (3.3 \times 0.02875) / 97.5$

$= 0.0000973\%\ ^{w}/_{v}$

$LOQ = 10\ \sigma / S$

$= (10 \times 0.02875) / 97.5$

$= 0.000295\%\ ^{w}/_{v}$

4.7.4.2 Linearity

Refer to Tables LLC, LLS and LLB

4.7.4 Precision

Refer to table PC to IMP

4.7.5 Stability studies of Ciprofloxacin and its Surrogates in solution

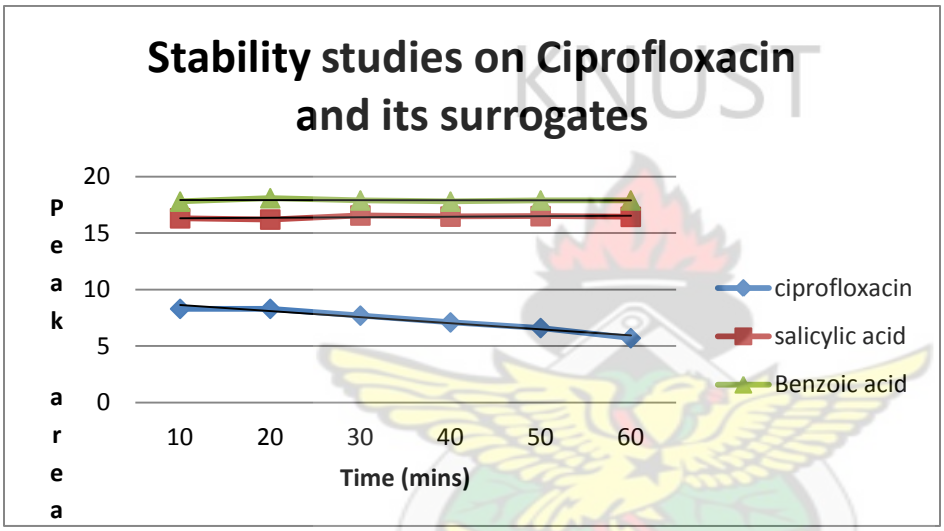

Figure 25: Stability studies on ciprofloxacin and surrogates in solution

4.8 Calculation of K values

4.8.1 Calculating K value for Ciprofloxacin with Salicylic acid as surrogate

Concentration of Ciprofloxacin, $C_{analyte}$ = 0.01%w/v

Concentration of Salicylic acid, $C_{Standard}$ = 0.01%w/v

Peak area Ciprofloxacin, $A_{analyte}$ = 11.94

Peak area of Salicylic acid, $A_{standard}$ = 25.74

$K = (A_{analyte} \times C_{standard}) / C_{analyte} \times A_{standard}$

= (11.94 x 0.01) / 0.01 x 25.74

= 0.4639

4.8.2 Calculating K value for Ciprofloxacin with Benzoic acid as surrogate

Concentration of analyte = 0.01%w/v

Concentration of standard = 0.01%w/v

Peak area Ciprofloxacin, = 6.18

Peak area of Benzoic acid, = 15.9

$K = (A_{analyte} \times C_{standard}) / C_{analyte} \times A_{standard}$

= (6.18 x 0.01) / (0.01 x 15.9)

= 0.3887

Table 13: K values determined for the use of salicylic and benzoic acid as surrogates in the analysis of Ciprofloxacin

Surrogate Standard	Calculated K value					
	1	2	3	4	5	Mean K
Salicylic acid	0.4619	0.4719	0.4527	0.4667	0.4477	0.4602±0.010
Benzoic acid	0.3967	0.3887	0.3961	0.3804	0.3988	0.3921± 0.008

4.9 Calculation for percentage contents

4.9.1 Calculating percentage of Ciprofloxacin in Ciprofloxacin tablet, UC using the K value from Benzoic acid

Average weight of Ciprofloxacin UC tablet = 0.8434g, which contains 0.5g or 500mg pure Ciprofloxacin

However, 84.40g was weighed containing 50.04mg or 0.05004g

Diluting this amount in 100ml yielded 0.05004%w/v. 1ml of this solution was further diluted to 0.005004%w/v. Similar dilutions were done to the pure Benzoic acid to obtain 0.005%w/v as the standard solution.

Peak area for Ciprofloxacin UC = 3.10, peak area for benzoic acid = 8.06

Concentration of analyte = (area of analyte x concentration of standard) / (k value x Area of standard)

Concentration of analyte = (3.10 x 0.005) / (0.3921 x 8.06)

= 0.004905%w/v

Percentage content = (Actual concentration/Nominal concentration) x 100

= (0.004905 / 0.005004) x 100

= 98.15%$^{w}/_{w}$

4.9.2 Calculating percentage of Ciprofloxacin in Ciprofloxacin tablet EC, using the K value from Salicylic acid

Average weight of content of EC tablet = 0.8234g, containing 0.5g or 500mg of Ciprofloxacin

Hence 0.05g of the active constituent is in = (0.05/0.5) x 0.8234

= 0.08234g

However 0.08240g was weighed, containing Ciprofloxacin of = (0.0824/0.08234) x 0.05

= 0.050036g

100ml dissolution of this yielded 0.050036%w/v. 1ml of this solution was further diluted to 0.0050036%w/v. salicylic acid was diluted to 0.005%w/v.

Peak area of Ciprofloxacin EC = 3.45

Peak area for salicylic acid = 7.6

Concentration of analyte = (Area of analyte x concentration of standard) / (k value x area of standard)

= (3.45 x 0.005) / (0.4602 x 7.62)

= 0.004919%w/v

Percentage content = (Actual concentration / Nominal concentration) x 100

= (0.0049190/ 0.0050036) x 100

= 98.31%$^{w}/_{w}$

Repeated calculations using the K values from salicylic and Benzoic acids for all the various brands of Ciprofloxacin tablets and infusion are provided.

Table 14: Mean percentage content for the Ciprofloxacin tablets analysed using the Surrogates

Surrogate	**Mean Percentage content of brand**				
	UC	MC	DC	SC	EC
Benzoic acid	99.99±0.53	95.90±0.48	97.85±0.62	99.06±0.64	96.79±0.62
Salicylic acid	100.35±0.34	96.90±0.19	96.50±0.27	97.81±0.50	95.83±0.68

Table 15: Mean percentage content for the Ciprofloxacin infusion analysed using the surrogates

Surrogate	Mean percentage content of brand			
	ECI	MCI	SCI	LCI
Salicylic acid	96.89±0.53	100.17±0.68	95.58±0.37	95.60±0.38
Benzoic acid	96.64±0.58	100.04±0.52	96.54±0.36	97.37±0.29

4.10 Comparing the Accuracy of the two Procedures using the t-Test

$t = (d / S_d) \times \sqrt{N}$

Where d and S_d are the mean and standard deviation of the difference between paired values.

N = number of values within the sets.

Meanwhile for five values, the degree of freedom is 4, with its critical value of "t" at the 95% confidence level for a two-tailed test being 2.78.

A sample calculation done using the standard method of determining the percentage content of Ciprofloxacin in UC and the developed method with salicylic acid is presented in the table below:

Table 16: Sample calculation for t

Percentage content of Ciprofloxacin Tablet (%)		
Standard method	New method	Standard method – New method
101.19	100.56	0.63
100.8	100.34	0.46
99.92	101.32	-1.4
101.36	99.6	1.76
99.85	98.15	1.7

Mean d =0.63

S_d =1.2818

$t = (d / S_d) \times \sqrt{N}$

= (0.63/1.2818) x √5

= 1.0990

Table 17: t-Test for UC

Standard procedure % $^{w}/_{w}$	New method-salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
101.19	100.56	100.23
100.8	100.34	99.81
99.92	101.32	101.3
101.36	99.6	100.92
99.85	98.15	99.5
	t= 1.0990	t= 0.6280

The t-values that were calculated for Ciprofloxacin brand UC, 1.0990 and 0.6280 fell below the critical value of 2.78 when Salicylic acid and Benzoic acid were employed as surrogate reference standards. This does not give enough evidence that there is significant difference between the two methods for analyzing brand UC. As such the null hypothesis is retained at 95% confidence level.

Table 18: t-Test for MC

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
96.85	97.2	96.91
97.72	94.32	97.19
95.34	95.54	97.35
94.72	96.21	96.82
97.69	96.23	96.21
	t= 0.6628	T= 0.6111

Table 19: t-Test for DC

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
94.25	98.41	95.95
95.93	98.32	96.29

96.67	96.79	96.73
95.32	96.12	96.08
96.97	99.6	97.43
	t= 2.8304	t= 2.3757

Table 20: t-Test for SC

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
99.24	96.68	98.65
98.88	99.65	97.37
99.54	100.48	98.97
100.23	99.54	96.18
99.02	98.93	97.9
	t= 0.5160	t= 2.4315

Table 21: t-Test for EC

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
97.14	96.31	95.19
97.65	98.32	98.31
96.54	98.06	95.29
98.26	96.35	96.08
96.58	94.91	94.28
	t= 0.6656	t= 2.5661

Table 22: t-Test for ECI

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
96.28	96.53	95.84
96.6	98.78	96.21
95.32	96.62	96.78
97.64	95.58	96.73
95.58	95.67	98.91
	t= 0.4944	t= 0.7706

Table 23: t-Test for MCI

Standard procedure % $^{w}/_{w}$	New method – salicylic acid % $^{w}/_{w}$	New method-benzoic acid % $^{w}/_{w}$
98.45	100.69	99.87

100.12	99.65	102.56
99.68	99.19	100.12
101.22	101.73	98.83
100.42	98.92	99.96
	t = 0.0919	t= 0.3508

.

Table 24: t-Test for SCI

Standard procedure $\%\ ^{w}/_{w}$	New method – salicylic acid $\%\ ^{w}/_{w}$	New method-benzoic acid $\%\ ^{w}/_{w}$
95.26	96.23	95.63
95.64	97.91	96.12
97.65	96.41	96.39
98.68	95.82	94.27
97.21	96.32	95.48
	t= 0.3914	t= 1.4731

Table 25: t-Test for LCI

Standard procedure $\%\ ^{w}/_{w}$	New method – salicylic acid $\%\ ^{w}/_{w}$	New method-benzoic acid $\%\ ^{w}/_{w}$
98.34	97.25	96.56
98.45	96.48	95.24
97.56	97.18	96.39
99.12	97.69	94.61
97.82	98.26	95.19
	t= 2.1098	t=4.5888

CHAPTER FIVE

5.0 DISCUSSION

5.0.1 Identification test and assay

The analyses of drugs in quality assurance are done in relation to reference standards. Since these standards serve as benchmark, their identification and purity is essential to the subsequent work.

In identification of Benzoic acid a clear colourless solution was observed in alcohol. Furthermore a solution of this produced a yellow precipitate upon the addition of ferric chloride solution which dissolved in ether. This was in compliance with the British Pharmacopoeia (BP). Benzoic acid had a percentage content of 99.30% which also fell within the permissible range of the BP, i. e 99% to 100.5%. Melting point determination of this sample was 123-125 ^{O}C as against the BP's range of 121-124 ^{O}C.[9] Considering these factors the sample was sufficiently pure for the analysis.

The British Pharmacopoeia states that the purity of Ciprofloxacin Hydrochloride should not be less than 98% and not more than the equivalent of 102%. An assay of this sample yielded 98.9%. This complies with the range provided by the British Pharmacopoeia. Subsequently its UV absorption spectrum gave absorption maxima of 274nm which is typical of Ciprofloxacin.[10][45] Coupled with this, in solution, a pH of 3.9 was observed for the clear solution which falls within the BP's range of 3.5 to 4.5.

Salicylic acid was assayed to obtain a percentage content of 99%. In comparison, the BP states that Salicylic acid should not be less than 99% and more than 100.5% as its percentage content. The melting point of salicylic acid sample ranged between 157-158^{o}c as against literature range

of 158 ^{o}c to 161 ^{o}c. In a basic solution of sodium hydroxide a violet colour was observed when ferric chloride was added, which persisted upon the addition of acetic acid. The sample therefore complied with the test for salicylates. These markers indicate that the sample was Salicylic acid and sufficiently pure for onward analysis.

Thin layer chromatography of the samples gave Rf values comparable with the Rf values of the reference standard. Whereas the tablets gave Rf values 0f 0.696, 0.70, 0.68, 0.684 and 0.690 for Ciprofloxacin tablets UC, MC, DC, SC and EC along with the reference standard, the infusions gave Rf values of 0.70, 0.682, 0.675 and 0.7 for brands ECI, MCI, SCI and LCI respectively.

5.0.2 Uniformity of Weight

According to the British Pharmacopoeia, not more than two of the individual weights should deviate by more than the figure provided for the respective weight in table 5.1 and none should also deviate by more than twice that percentage. [9]

Table 26: Uniformity of weight of tablets (uncoated and film coated)

Average weight of tablet	Percentage Deviation allowed	Number of tablets
80mg or less	±10	20
More than 80mg and less than 250mg	±7.5	20
250mg or more	±5	20

Source: BP 2007

In effect not more than two should deviate by 5% and none should deviate by more than 10% for tablets weighing 250mg or more. The total weight of the tablets were 16.8676g, 15.0043g, 16.4688g, 18.9651g and 19.1299g for brands DC, SC, EC, UC and SC. Meanwhile brand DC had an average of 0.8434g. Even though, one of the tablets deviated by more than 5.4446%, this

brand still passed for the uniformity of weight because the value was not more than 10%. Similarly, for brand SC with an average of 0.7502g, none of the tablets deviated by more than 10%. However, it failed in the Uniformity of weight since three of the tablets deviated by 7.533%, 9.6887% and 5.4672. Brand EC with an average of 0.8234g passed for the uniformity of weight with the highest deviation being 1.6565g. Likewise UC and SC with averages of 0.9483 and 0.9565 passed, with their highest deviations being1.9462 and 2.4688 respectively.

5.0.3 Stability Studies on drug samples in Solution

Injections were done for the drug samples six times at 10minutes interval to monitor the stability of these compounds as per the experiment. Peak areas were then plotted against the time. The graph showed a linear plot with reference to the X-axis for Salicylic acid and Benzoic acid. However, the line for ciprofloxacin showed a negative slope from the 20th minute. As such Ciprofloxacin had to be injected as quickly as possible and within 20minutes.

5.0.4 Determination of Percentage Content of Ciprofloxacin Tablets

The five brands of the Ciprofloxacin tablets were analysed to obtain their percentage contents prior to the method development. This was carried out to give a fair idea of the purity of the commercial samples so that results from the developed method could be compared.

In a the assay of Ciprofloxacin tablets, brands UC and MC had percentage contents of 100.62% and 96.47%, whiles DC, SC and EC had percentage contents of 95.83%, 99.38% and 97.23% respectively. The British Pharmacopoeia indicates that the percentage contents of Ciprofloxacin tablets should not be less than 95% and not more than 105%. As such all the commercial samples passed the test.

5.0.5 Determination of Percentage Content of Ciprofloxacin Infusion

Similarly the percentage content for the various brands of the Ciprofloxacin infusions was determined. The four brands namely ECI, MCI, SCI and LCI had percentage contents of 96.28%, 99.98%, 96.89% and 98.26%. Similar to the Ciprofloxacin tablets the percentage content stipulated by the British Pharmacopoeia is a range of 95% to 100.5% of the stated amount. In effect all the commercial samples fell within the BP's range.

5.0.6 HPLC Method Development

Method development involves trial and error to some extent. However, one needs to get a fair idea of the molecular weight, polarity, ionic character, the nature of the sample and the solubility of the compound being analysed. This research seeks to develop a method that would be rapid, accurate and precise for analyzing Ciprofloxacin. As such a factor like pH of the mobile phase which has a great effect on the elution of a compound that is ionizable needed to be considered. Usually the retention time decreases with increasing pH, for acids. Thus the elution strength of the mobile phase depends on the polarity. The higher the polarity, the higher the elution strength. Meanwhile the effect of a buffer with a very low pH on the column was also considered for the compounds. In a typical reverse phase chromatography, as was employed for this research, hydrophilic compounds were separated using polar solvents of varying compositions to acquire the best resolution.

The analyses of Ciprofloxacin tablets and infusions were done using a Bondclone 10 Phenomenex C18 300 x 3.90mm column. This was in line with the reverse phase that was to be employed. The polar mobile phase employed was water and methanol at different combinations,

some of which yielded broad and delayed peaks. Coupled with the introduction of a surrogate the resolution of the peaks became poorer. Since changes in the ionization state of solutes affects the interaction between the solute and stationary phase, a buffer was added to control the pH of the mobile phase.

The best results were observed with a mobile phase combination of 50:45:5 methanol, water and phosphate buffer of pH-3. The initial amount of a smaller percentage of methanol yielded a higher retention time, prompting the increase to 50%, which provided a shorter time of analysis. The analysis then proceeded with the introduction of other compounds to act as surrogates. Salicylic acid and Benzoic Acid were chosen due to the better resolution that was obtained on each occasion.

Because of the nature of the compound, availability and sensitivity of the UV detector the UV spectrometer was chosen. In other to identify a suitable wavelength for the analysis a scan was taken during the analysis of Ciprofloxacin with the HPLC. Based on this scan the research was under taken at a wavelength of 239nm.

Under these circumstances the retention times for Ciprofloxacin, Salicylic acid and Benzoic acid were 2.68 ± 0.20 minutes, 4.69 ±0.20 minutes and 4.44 ± 0.15 minutes. Even though Ciprofloxacin has a bulkier quinoline ring which induces non-polarity, the least time of interaction for Ciprofloxacin could be attributed to the ionization of the drug in the mobile phase as compared to Benzoic acid and Salicylic acid, since very polar molecules have less interaction with the non-polar stationary phase. Benzoic acid and Salicylic acid both have benzene rings which brings a degree of non-polarity. But an additional –OH group to the –COOH group could

increase the polarity in Salicylic acid. Since this situation reduces its interaction with the stationary phase Salicylic acid had a smaller retention time.

5.0.7 Limit of Detection and Quantitation (LOD and LOQ)

This is the lowest concentration that can be detected for a particular sample within the conditions that the experiment was conducted. For Ciprofloxacin the limit of detection was found to be 0.0000973%$^{w}/_{v}$, while Salicylic acid and Benzoic acid had limits of detections of 0.000235 %$^{w}/_{v}$ and 0.0002987%$^{w}/_{v}$ respectively.

The limit of quantitation is the lowest concentration of the sample that can be determined with acceptable precision and accuracy. For Ciprofloxacin LOQ was calculated and found to be 0.000295%$^{w}/_{v}$ whiles the LOQ for Salicylic acid and Benzoic acid were found as 0.000707%$^{w}/_{v}$ and 0.000870%$^{w}/_{v}$ respectively.

5.0.8 Linearity

The linearity of a procedure is its ability to get results that are directly proportional to the concentration within a given range. A correlation coefficient of ≥ 0.99 indicates an appreciable linearity. In each case the linearity was above 0.99 and as such the method gives a good level of linearity.

5.0.9 Robustness

There were variations in certain factors such as the flow rate from 1.5ml/min to 1.4ml/min and 1.6. This did not present a significant difference in the results that were obtained. Similarly, a slight difference in wavelength of absorption from 239nm to 238nm did not bring a significant

difference. The method was also observed to be robust for a variation in the mobile phase such as 5% increase in methanol.

5.0.10 Relative Standard Deviation and Precision

In other to show repeatability and intermediate precision the relative standard deviations of the percentage contents were calculated. Guidelines indicate that the RSD should not be more than 2%.[47] RSD of the samples from tables PC to IPS were all below this value. This indicates a good precision for the various concentrations of the analyte and the surrogates.

5.0.11 Determination of the Constant K

K is the constant that relates the concentration of the analyte to the peak area for that analyte. This value is not proportional to the concentrations of the samples since a change in concentration rather affects a change in the peak area. As such the calculated K at each instance could be expected to be the same. The various concentrations and their corresponding peak areas were substituted into the formula for the determination of the constant K. The K values were then determined. However, there were some variations in these values, which were not significant. This could much be attributed to random errors form the statistical data. Table 13 in page 68 presents these figures.

5.0.12 Determinations of Percentage content using the constant K

The various brands of the Ciprofloxacin tablets and infusions were also assayed using the method developed. Usually pure reference samples are employed in the development of new methods and then actual formulations are subjected to these conditions for analysis. The results obtained after using the K values for each of the surrogate reference standards have been

presented in tables 14 and 15. The peak areas and the various concentrations employed are provided in the appendix from table PC.1 to PC.16.

The British Pharmacopoeia states the range of Ciprofloxacin tablets and intravenous infusions to be between 95.0% - 105.0%.[9] The percentage content of all the brands that were analyzed using these surrogate reference standards fell within the range provided by the British Pharmacopoeia.

5.0.13 Comparing the Developed Method with the Standard method using

5.0.13.1 Ciprofloxacin Tablets

It is important for an analytical procedure to be free from systematic errors and as such the value obtained after an analysis should be checked for its closeness to the true value. However, random errors make it very unlikely that acquired value would be exactly the same as the standard value. In other to know whether the difference between the two procedures could be merely due to random errors requires the significance test known as the t-Test. If the absolute value of the t-value, which is from the calculation, is smaller than the critical value then the null hypothesis is retained. In this instance there is no evidence of systematic error. [48]

The null hypothesis indicates that the means of the two methods do not differ significantly at the 95% confidence level. For four degrees of freedom, the critical value of "t" at 95% confidence level is 2.78. [48]

The t_{exp} i.e. t value from the calculation for Ciprofloxacin tablet UC were 1.099 and 0.628 for the use of Salicylic acid and Benzoic acid as surrogates as compared to the standard method. For tablet MC similar calculations yielded 0.6628 and 0.6111 when the two surrogate procedures were compared with the standard method. Inferring from these results the null hypothesis is retained, and as such there is no evidence of systematic errors.

Meanwhile, the use of Salicylic acid and Benzoic acid as surrogates for analyzing tablet DC gave 2.8304 and 2.3757. The first value of t_{exp} is greater than the critical value at 95% confidence level. This indicates that the null hypothesis for the use of Salicylic acid as surrogate is rejected since there is an evidence of systematic error.

For tablets SC and EC the use of Salicylic and Benzoic acid as surrogates gave t_{exp} of 0.5160 and 2.4315, and then 0.6656 and 2.5661 respectively. Since none of these values is greater than the critical value of 2.78 the null hypothesis is retained, indicating that there is no evidence of systematic error.

5.0.13.2 Ciprofloxacin Infusions

The t_{exp} for Ciprofloxacin infusion ECI were 0.4944 and 0.7706 for the use of Salicylic and Benzoic acids in comparison with the standard method of analyzing Ciprofloxacin. In the case of brand MCI the t_{exp} were 0.0919 and 0.3508 for Salicylic acid and Benzoic acid surrogate reference standards. Subsequently for SCI the t_{exp} were 0.3914 and 1.4731 for Salicylic acid and Benzoic acid. These values are all less than the critical value of 2.78 and hence the null hypothesis is retained. However, the t_{exp} for infusion LCI were 2.1098 for Salicylic acid and 4.5888 for Benzoic acid, higher than the critical value. Thus the null hypothesis is rejected for the use of Benzoic acid in analyzing LCI, even though the percentage contents determined by both methods fell within the range of the BP. This indicates that there is a significant difference between the means of the two methods.

5.1 Conclusion

In a High Performance Liquid Chromatographic analysis of Ciprofloxacin, Salicylic acid and Benzoic acid were identified as Surrogate reference Standards. In effect correction factors for these chemicals were established with the use of a mobile phase combination of methanol, water

and phosphate buffer pH-3 at a ratio of 50:45:5. Salicylic acid gave a value of 0.4602 ± 0.010 while Benzoic acid gave 0.3921 ± 0.008 as K.

Results from the analysis suggest that Salicylic acid and Benzoic acid can be used for the analysis of Ciprofloxacin in the absence of pure reference standard. This method of assay is cheaper, simple, precise and reliable. Based upon its validation this procedure is suitable for the assay of Ciprofloxacin tablet and infusion.

5.2 Recommendations

The search for other drugs as surrogate reference standards should be encouraged for the analysis of other drugs. This will reduce difficulties that academic institutions and regulatory bodies go through in search of pure reference standards for analysis.

Further work should also be done to establish whether the presence of excipients will have a significant effect on the Value of K.

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APPENDIX

P. 1 Preparation of solutions

P.1.1 Preparation of 0.1M Perchloric acid

8.5ml of 72% perchloric acid was added to 900ml glacial acetic acid with continuous swirling gradually. 30ml of acetic anhydride was then added to the solution slowly to ensure efficient mixing and the volume adjusted to 1L with glacial acetic acid. The solution was then allowed to stand for 24hrs before use.

P.1.2 Preparation of 0.1M NaOH

Molecular mass = 40g/ml assay = 98%

40g of NaOH in 1000ml ≡ 1M NaOH

4g of NaOH in 1000ml ≡ 0.1M NaOH

0.4g of NaOH in 100ml ≡ 0.1M NaOH

But from assay 98% ≡ 0.4g

100% ≡ (0.4g/98) x 100 = 0.4081g of NaOH

0.4081g of sodium hydroxide pellets were weighed into a beaker and dissolved with distilled water gradually to complete dissolution. The solution was then filtered into a 100ml volumetric flask using a whatman filter paper. After shaken to ensure dissolution it was made up to the mark with the water.

Uniformity of Weight for Ciprofloxacin tablets

Table U1: Uniformity of Weight, DC

Ciprofloxacin Tablets 500mg
Name of manufacturer: Danadams Pharmaceuticals Ltd, Ghana
Batch Number: 12050905
Weight of 20 tablets: 16.8676g
Average weight: 0.8434g

Number	**Weight of tablet**	**Deviation**	**% Deviation**
1	0.829	-0.0144	-1.7050
2	0.8137	-0.0297	-3.5191
3	0.8221	-0.0213	-2.5231
4	0.8455	0.0021	0.2514
5	0.8316	-0.0118	-1.3967
6	0.8344	-0.0090	-1.0647
7	0.8504	0.0070	0.8323
8	0.8675	0.0241	2.8599
9	0.8422	-0.0012	-0.1399
10	0.8434	0.0000	0.0024
11	0.8432	-0.0002	-0.0213
12	0.8534	0.0100	1.1880
13	0.8451	0.0017	0.2039
14	0.8412	-0.0022	-0.2585
15	0.8418	-0.0016	-0.1873
16	0.8365	-0.0069	-0.8157
17	0.8893	0.0459	5.4446
18	0.8416	-0.0018	-0.2111
19	0.8211	-0.0223	-2.6417
20	0.8746	0.0312	3.7017

Table U2: Uniformity of weight, SC

Ciprofloxacin Tablets 500mg			
Name of manufacturer: Sravani Laboratories Ltd, India			
Batch Number: CP-01			
Weight of 20 tablets: 15.0043g			
Number	**Weight of tablet**	**Deviation**	**% Deviation**
1	0.723	-0.0272	-3.6277
2	0.738	-0.0122	-1.6282
3	0.7562	0.0060	0.7978
4	0.7475	-0.0027	-0.3619
5	0.7744	0.0242	3.2238
6	0.7369	-0.0133	-1.7749
7	0.7351	-0.0151	-2.0148
8	0.7739	0.0237	3.1572
9	0.7495	-0.0007	-0.0953
10	0.756	0.0058	0.7711
11	0.7092	-0.0410	-5.4672
12	0.7251	-0.0251	-3.3478
13	0.7654	0.0152	2.0241
14	0.6937	-0.0565	-7.5333
15	0.7616	0.0114	1.5176
16	0.8229	0.0727	9.6887
17	0.7381	-0.0121	-1.6149
18	0.7591	0.0089	1.1844
19	0.7648	0.0146	1.9441
20	0.7739	0.0237	3.1572

Table U3: Uniformity of weight, EC

Ciprofloxacin Tablets 500mg
Name of manufacturer: Ernest Chemist Ltd, Ghana
Batch Number: 1011L
Weight of 20 tablets: 16.4688g
Average weight: 0.8234g

Number	**Weight of tablet**	**Deviation**	**% Deviation**
1	0.8187	-0.0047	-0.5756
2	0.8212	-0.0022	-0.2720
3	0.8217	-0.0017	-0.2113
4	0.8265	0.0031	0.3716
5	0.8249	0.0015	0.1773
6	0.8353	0.0119	1.4403
7	0.8289	0.0055	0.6631
8	0.8098	-0.0136	-1.6565
9	0.8100	-0.0134	-1.6322
10	0.8310	0.0076	0.9181
11	0.8168	-0.0066	-0.8064
12	0.8306	0.0072	0.8695
13	0.8249	0.0015	0.1773
14	0.8136	-0.0098	-1.1950
15	0.8262	0.0028	0.3352
16	0.8262	0.0028	0.3352
17	0.8209	-0.0025	-0.3085
18	0.8283	0.0049	0.5902
19	0.8193	-0.0041	-0.5028
20	0.8340	0.0106	1.2824

Table U4: Uniformity of weight for UC

Ciprofloxacin Tablets 500mg
Name of manufacturer: Unichem Industries Ltd. Ghana
Batch Number: 11
Weight of 20 tablets: 18.9651g
Average weight: 0.9483g

Number	**Weight of tablet**	**Deviation**	**% Deviation**
1	0.9555	0.0072	0.7640
2	0.9413	-0.0070	-0.7335
3	0.9429	-0.0054	-0.5647
4	0.9483	0.0000	0.0047
5	0.9467	-0.0016	-0.1640
6	0.9546	0.0063	0.6691
7	0.9441	-0.0042	-0.4382
8	0.956	0.0077	0.8168
9	0.9453	-0.0030	-0.3116
10	0.9573	0.0090	0.9539
11	0.9298	-0.0185	-1.9462
12	0.9614	0.0131	1.3862
13	0.9455	-0.0028	-0.2905
14	0.9431	-0.0052	-0.5436
15	0.9529	0.0046	0.4898
16	0.9599	0.0116	1.2280
17	0.9463	-0.0020	-0.2062
18	0.9434	-0.0049	-0.5120
19	0.9552	0.0069	0.7324
20	0.9356	-0.0127	-1.3346

Table U5: Uniformity of weight for SC

Ciprofloxacin Tablets 500mg
Name of manufacturer: Mission Vivacare Ltd, India
Batch Number: PC2111001A
Weight of 20 tablets: 19.1299 g
Average weight: 0.9565g

Number	**Weight of tablet**	**Deviation**	**% Deviation**
1	0.9549	-0.0016	-0.1668
2	0.9496	-0.0069	-0.7209
3	0.9729	0.0164	1.7151
4	0.9525	-0.0040	-0.4177
5	0.9676	0.0111	1.1610
6	0.9646	0.0081	0.8474
7	0.9647	0.0082	0.8578
8	0.9803	0.0238	2.4888
9	0.9545	-0.0020	-0.2086
10	0.957	0.0005	0.0528
11	0.9549	-0.0016	-0.1668
12	0.9538	-0.0027	-0.2818
13	0.9395	-0.0170	-1.7768
14	0.9503	-0.0062	-0.6477
15	0.9709	0.0144	1.5060
16	0.933	-0.0235	-2.4564
17	0.9565	0.0000	0.0005
18	0.9534	-0.0031	-0.3236
19	0.9503	-0.0062	-0.6477
20	0.9487	-0.0078	-0.8150

Table LDC: Limit of detection and Quantitation for Ciprofloxacin and the Surrogates

Sample	LOD	LOQ
Ciprofloxacin	0.0000973	0.000295
Salicylic acid	0.000233	0.000707
Benzoic acid	0.000287	0.000870

Linearity for Ciprofloxacin and the surrogates

Table LLC: Linearity for Ciprofloxacin

Range of concentration: 0.002 – 0.01%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 975x + 4.25	0.997

Table LLS: Linearity of Salicylic acid

Range of concentration: 0.0002 – 0.001%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 1595x + 5.11	0.995

Table LLB: Linearity of Benzoic acid

Range of concentration: 0.0002 – 0.001%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 2143x + 4.50	0.996

Repeatability

Table PC: Ciprofloxacin

Relative Standard Deviation of Pure Salicylic acid Number of injections = 5								
Concentration (%w/v)	**Peak Area**					**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**			
0.004	11.82	11.91	11.88	11.98	11.75	11.868	0.087579	0.73794
0.002	7.96	8.08	8.1	7.99	8.15	8.056	0.07893	0.97977
0.01	20.5	21.2	20.81	21.16	21.09	20.952	0.295076	1.40834

Table PS: Salicylic acid

Relative Standard Deviation of Pure Ciprofloxacin, Number of injections = 5								
Concentration (%w/v)	**Peak Area**					**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**			
0.01	13.47	13.61	14.1	13.83	13.98	13.798	0.258979	1.87693
0.006	10.31	10.17	9.97	10.28	9.95	10.136	0.169056	1.66788

Table PB: Benzoic acid

Relative Standard Deviation of Pure Ciprofloxacin, Number of injections = 5								
Concentration (%w/v)	**Peak Area**					**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**			
0.008	21.84	22.08	21.95	22.1	22.26	22.046	0.15931	0.72263
0.005	16.69	16.17	16.24	16.43	15.96	16.298	0.27617	1.69450

Table IPB: Intermediate Precision with Benzoic acid

	1	**2**	**3**	**4**	**5**	**6**	**Mean %**	**Standard Deviation**	**RSD (%)**
Day 1	98.71	99.81	99.35	97.43	100.04	99.12	99.07	0.936839	0.94557
Day 2	99.92	100.09	97.32	99.03	98.65	98.06	98.85	1.069556	1.08205
Day 3	98.02	98.73	97.59	96.66	98.71	99.26	98.16	0.941476	0.95911

Table IPS: Intermediate Precision with Salicylic acid

	1	**2**	**3**	**4**	**5**	**6**	**Mean %**	**Standard Deviation**	**RSD (%)**
Day 1	101.21	100.13	98.65	99.29	98.46	98.06	99.30	1.183453	1.19180
Day 2	98.36	98.95	100.27	97.81	99.52	98.91	98.97	0.861882	0.87085
Day 3	99.32	99.98	98.49	101.91	100.97	100.04	100.12	1.204117	1.20269

Robustness

Table RF : Variations in Flowrate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1.4	3	297.3	99.1	0.7033
1.5	3	297.38	99.12667	0.018633
1.6	3	297.49	99.16333	3.392133

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006066667	2	0.003033	0.002212	0.997791	5.143253
Within Groups	8.228133333	6	1.371356			
Total	8.2342	8				

Table RW: Variation in Wavelength

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	296.43	98.81	0.1996
Row 2	3	298.01	99.33667	0.660433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.416067	1	0.416067	0.967559	0.38098	7.708647
Within Groups	1.720067	4	0.430017			
Total	2.136133	5				

Titration Tables

Table T.1 Standardization of Sodium hydroxide with Sulphamic acid

Burrete reading(ml)	1st determination	2nd Determination
Final reading	26.2	26.1
Initial reading	0.0	0.0
Titre value	26.2	26.1

Average titre = (26.2 +26.1)/2

= 26.15ml

Table T.2 Standardization of Perchloric acid with Potassium Hydrogen Phthalate

Burrete reading(ml)	1st determination	2nd Determination	3rd Determination
Final reading	26.2	26.0	25.5
Initial reading	0.0	0.0	0.0
Titre value	26.2	26.0	25.5

Average titre = (26.2 + 26.0 + 25.5) / 3

= 25.9ml

Titre = 25.9 - 0.1(blank) = 25.8ml

Table T.3 Assay of Benzoic acid

Burrete reading/ml	1st Determination(0.2013g)	2nd Determination(0.2056g)
Final reading	17.1	17.3
Initial reading	0.0	0.0
Titre value	17.1	17.3

Table T.4 Assay of Salicylic acid

Burrete reading/ml	1st determination(0.1198g)	2nd determination(0.1184g)
Final reading	8.9	17.7
Initial reading	0.0	8.9
Titre value	8.9	8.8

Table T.5 Assay of Ciprofloxacin

Burrete reading/ml	1st determination(0.2071g)	2nd determination(0.2093g)	Blank
Final reading	6.2	12.3	0.4
Initial reading	0.0	6.2	0.0
Titre value	6.2	6.1	0.4

Determination of the constant K for Ciprofloxacin using

Table K.1 K values using Salicylic acid as the surrogate standard.

Concentration of Ciprofloxacin	**Peak Area of Ciprofloxacin**	**Peak Area of Salicylic acid**	**Concentration of Salicylic acid**	**K value**
0.01	11.94	25.85	0.01	0.4619
0.008	9.59	20.32	0.008	0.4719
0.002	2.39	5.28	0.002	0.4527
0.002	2.24	3.60	0.0015	0.4667
0.0015	1.82	2.71	0.001	0.4477
Average K = 0.4602 ± 0.010				

Table K.2 K values using Benzoic acid as the surrogate standard

Concentration of Ciprofloxacin	**Peak area Ciprofloxacin**	**Peak area Benzoic acid**	**Concentration of Benzoic acid**	**K value**
0.01	6.18	15.9	0.01	0.3887
0.008	4.99	12.58	0.008	0.3967
0.002	1.22	3.08	0.002	0.3961

0.001	0.65	1.63	0.001	0.3988
0.0015	0.93	4.89	0.003	0.3804
Average K= 0.3921 ± 0.008				

Determination of Percentage content of Ciprofloxacin in Ciprofloxacin Tablet Using the K

Table PC.1 Percentage content of Ciprofloxacin UC using salicylic acid as surrogate

K = 0.4602					
Ciprofloxacin tablet UC,0.5g , average weight = 0.9483g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	5.38	0.00496	2.475876	0.002003	100.23
0.002	5.46	0.00502	2.512692	0.001998	99.81
0.0015	3.88	0.002715	1.785576	0.001521	101.3
0.008	20.15	0.01872	9.27303	0.002019	100.92
0.001	2.88	0.00198	1.325376	0.001494	99.5

Cs is the concentration of the surrogate, As is the peak area for the surrogate.

Table PC.2 Percentage content of Ciprofloxacin MC using Salicylic acid as surrogate

K = 0.4602					
Ciprofloxacin tablet MC,0.5g , average weight = 0.9565g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	5.65	0.00504	2.60013	0.00193836	96.91
0.002	5.75	0.00514	2.64615	0.00194244	97.19
0.0015	4.51	0.00303	2.075502	0.00145989	97.35
0.008	21.55	0.0192	9.91731	0.00193601	96.82
0.001	2.99	0.00199	1.375998	0.00144622	96.21

Cs is the concentration of the surrogate, As is the peak area for the surrogate.

Table PC. 3 Percentage content of Ciprofloxacin Tablet DC using Salicylic acid

K = 0.4602					
Ciprofloxacin tablet DC,0.5g , average weight = 0.8434g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	5.53	0.00488	2.544906	0.00191756	95.95
0.002	5.51	0.0049	2.535702	0.0019324	96.29
0.0015	3.57	0.002385	1.642914	0.00145169	96.73
0.008	19.99	0.01768	9.199398	0.00192186	96.08

0.001	2.76	0.00186	1.270152	0.00146439	97.43

Cs is the concentration of the surrogate, As is the peak area for the surrogate.

Table PC. 4 Percentage content of Ciprofloxacin Tablet SC using Salicylic acid

K = 0.4602					
Ciprofloxacin tablet SC,0.5g , average weight = 0.7502g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	4.97	0.00452	2.287194	0.00197622	98.65
0.002	5.16	0.00462	2.374632	0.00194556	97.37
0.0015	3.71	0.002535	1.707342	0.00148476	98.97
0.008	20.69	0.01832	9.521538	0.00192406	96.18
0.001	2.69	0.00182	1.237938	0.00147019	97.9

Table PC.5 Percentage content of Ciprofloxacin Tablet UC using Benzoic acid as surrogate

K = 0.3921					
Ciprofloxacin tablet UC,0.5g , average weight = 0.9483g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	4.79	0.00567	1.878159	0.003019	100.56
0.002	3.00	0.00236	1.1763	0.002006	100.34
0.001	1.61	0.00064	0.631281	0.001014	101.32
0.008	12.70	0.00496	4.97967	0.000996	99.60
0.001	1.58	0.00487	0.619518	0.007861	98.15

Cs is the concentration of the surrogate, As is the peak area for the surrogate.

Table PC.6 Percentage content of Ciprofloxacin Tablet MC using Benzoic acid as surrogate

K = 0.3921					
Ciprofloxacin tablet MC,0.5g , average weight = 0.9565g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	4.83	0.00552	1.893843	0.00291471	97.2
0.002	2.92	0.00216	1.144932	0.00188657	94.32
0.001	1.6	0.0006	0.62736	0.00095639	95.54
0.008	12.51	0.00472	4.905171	0.00096225	96.21
0.001	1.62	0.00489	0.635202	0.00769834	96.23

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.7 Percentage content of ciprofloxacin Tablet DC using Benzoic acid

K = 0.3921					
Ciprofloxacin tablet DC,0.5g , average weight = 0.8434g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	4.54	0.00537	1.780134	0.00294922	98.41
0.002	2.76	0.00222	1.082196	0.00197746	98.32
0.001	1.58	0.00065	0.619518	0.00096849	96.79
0.008	12.52	0.00496	4.909092	0.00096148	96.12
0.001	1.56	0.00485	0.611676	0.00796173	99.6

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.8 Percentage content of Ciprofloxacin Tablet EC using Benzoic acid

K = 0.3921					
Ciprofloxacin tablet EC,0.5g , average weight = 0.8234g					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	4.74	0.00537	1.858554	0.00288934	96.31
0.002	2.88	0.00222	1.129248	0.00196591	98.32
0.001	1.69	0.00065	0.662649	0.00098091	98.06
0.008	13.13	0.00496	5.148273	0.00096343	96.35
0.001	1.63	0.00485	0.639123	0.00758852	94.91

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.9 Percentage content of Ciprofloxacin infusion ECI using Benzoic acid

K = 0.3921					
Ciprofloxacin intravenous infusion ECI,200mg/100ml g ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	4.78	0.00543	1.874238	0.002897	96.53
0.002	2.92	0.00226	1.144932	0.001974	98.78
0.001	1.74	0.00066	0.682254	0.000967	96.62
0.008	13.24	0.00496	5.191404	0.000955	95.58
0.001	1.61	0.00483	0.631281	0.007651	95.67

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.10 Percentage content of Ciprofloxacin infusion MCI using Benzoic acid

K = 0.3921					
Ciprofloxacin intravenous infusion MCI, 200mg/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	5.04	0.00597	1.976184	0.003021	100.6991
0.002	2.92	0.00228	1.144932	0.001991	99.65
0.001	1.82	0.00071	0.713622	0.000995	99.19
0.008	13.25	0.00528	5.195325	0.001016	101.73
0.001	1.83	0.00568	0.717543	0.007916	98.92

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.11 Percentage content of Ciprofloxacin infusion SCI using Benzoic acid

K = 0.3921					
Ciprofloxacin intravenous infusion SCI, 200mg/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	5.17	0.00585	2.027157	0.002886	96.23
0.002	2.91	0.00224	1.141011	0.001963	97.91
0.001	1.85	0.0007	0.725385	0.000965	96.41
0.008	13.2	0.00496	5.17572	0.000958	95.82
0.001	1.83	0.00553	0.717543	0.007707	96.32

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.12 Percentage content of Ciprofloxacin infusion LCI using Benzoic acid

K = 0.3921					
Ciprofloxacin intravenous infusion LCI, 200mg100/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.003	5.19	0.00594	2.034999	0.002919	97.25
0.002	2.98	0.00226	1.168458	0.001934	96.48
0.001	1.89	0.00072	0.741069	0.000972	97.18
0.008	13.2	0.00504	5.17572	0.000974	97.69
0.001	1.82	0.00561	0.713622	0.007861	98.26

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.13 Percentage content of Ciprofloxacin infusion ECI using Salicylic acid

K = 0.4602					
Ciprofloxacin intravenous infusion ECI, 200mg100/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	5.66	0.005	2.604732	0.00192	95.84
0.002	5.67	0.00502	2.609334	0.001924	96.21
0.0015	4.43	0.002955	2.038686	0.001449	96.78
0.008	21.48	0.01912	9.885096	0.001934	96.73
0.001	2.9	0.00198	1.33458	0.001484	98.91

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.14 Percentage content of Ciprofloxacin infusion MCI using Salicylic acid

K = 0.4602					
Ciprofloxacin intravenous infusion MCI, 200mg100/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	5.61	0.00516	2.581722	0.001999	99.87
0.002	5.65	0.00534	2.60013	0.002054	102.56
0.0015	4.43	0.00306	2.038686	0.001501	100.12
0.008	21.04	0.01904	9.682608	0.001966	98.33
0.001	2.86	0.00197	1.316172	0.001497	99.96

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.15 Percentage content of Ciprofloxacin infusion SCI using Salicylic acid

K = 0.4602					
Ciprofloxacin intravenous infusion SCI, 200mg100/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	6.34	0.00558	2.917668	0.001912	95.63
0.002	6.24	0.00552	2.871648	0.001922	96.12
0.0015	4.62	0.003075	2.126124	0.001446	96.39
0.008	24.06	0.02088	11.07241	0.001886	94.27
0.001	3.02	0.00199	1.389804	0.001432	95.48

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Table PC.16 Percentage content of Ciprofloxacin infusion LCI using Salicylic acid

K = 0.4602					
Ciprofloxacin intravenous infusion LCI, 200mg100/ml ,					
Cs	As	Aa x Cs	K x As	Aa x Cs/ K x As	Percentage
0.002	6.28	0.00558	2.890056	0.001931	96.56
0.002	6.18	0.00542	2.844036	0.001906	95.24
0.0015	4.58	0.003045	2.107716	0.001445	96.39
0.008	24.14	0.02104	11.10923	0.001894	94.61
0.001	3.09	0.00203	1.422018	0.001428	95.19

Cs is the concentration of the surrogate, As is the peak area for the surrogate

Fig 26: UV-Visible Spectrum of Ciprofloxacin