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SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY; A CASE STUDY OF THE ANALYSIS OF PIROXICAM CAPSULES AND METRONIDAZOLE TABLETS

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DECLARATION

It is hereby declared that this thesis is the outcome of research work undertaken by the author. Any assistance obtained has been duly acknowledged. The work has neither in part nor wholly been presented for another degree elsewhere.

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DEDICATION

This project is dedicated to my dear mother, all my brothers and also to the memory of my late father.

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I would first of all give thanks to the almighty God for his continual guidance and protection which has sustained me to this level.

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ABSTRACT

A simple, rapid, accurate and sensitive High Performance Liquid Chromatography (HPLC) method was developed for quantitative analysis of Piroxicam and Metronidazole in Piroxicam capsules and Metronidazole tablets using surrogate reference standards with UV detection. Metronidazole, Metformin and Ascorbic acid pure samples were used as surrogate reference standards for the analysis of Piroxicam. The chromatography parameters were stainless steel ODS C18 Phenomenex 250 x 4.6mm column particle size. The isocratic mobile phase was methanol:phosphate buffer pH 6.2 (50:50; v/v) at a flow rate of at 1.0 $mLmin^{-1}$. The determinations were performed using UV-Vis detector set at 254 nm. Mean retention times (minutes) of 6.83±0.065, 4.07±0.058, 3.42±0.049 and 2.56±0.055 were recorded for Piroxicam, Metronidazole, Metformin and Ascorbic acid respectively. A constant, K, was determined for Piroxicam using each of the surrogate reference standards. The respective K values obtained when Metronidazole, Metformin and Ascorbic acid were used as surrogate reference standards were 1.9411± 0.004, 1.6200 ± 0.006 and 0.4569 ± 0.006. Ascorbic acid and Para aminophenol pure samples were used as surrogate reference standards for the analysis of Metronidazole. The chromatography parameters were stainless steel ODS C18 Phenomenex 250 x 4.6mm column particle size. The isocratic mobile phase was methanol:phosphate buffer pH 6.2 (50:50; v/v) at a flow rate of at 1.5 $mLmin^{-1}$. The determinations were performed using UV-Vis detector set at 254 nm. Mean retention times (minutes) of 4.24±0.014, 1.80±0.012 and 2.82±0.015 were recorded for Metronidazole, Ascorbic acid and Para aminophenol respectively. A constant, K, was determined for Metronidazole using each of the surrogate reference standards. The respective K values obtained when Ascorbic acid and Para aminophenol were used as surrogate reference standards were 0.6763 ± 0.004 and 1.1229 ± 0.016. The results obtained when the

methods were used for the analysis of the Piroxicam capsules and Metronidazole tablets were comparable to standard methods of analysis stated in the BP and USP respectively. The method showed adequate precision, with a relative standard deviation (RSD) less than 2% in each case. The HPLC methods developed in this study showed specificity and selectivity with linearity in the working range and good precision and accuracy, making them very suitable for quantification of Piroxicam capsules and Metronidazole tablets.

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ABBREVIATIONS

BP: British Pharmacopoeia

CQA: Critical Quality Attribute

ECL: Ernest Chemist Ltd, Ghana

FDA: Food and Drug Administration

FTIR: Fourier Transform Infrared

GC: Gas Chromatography

GLP: Good Laboratory Practice

GMP: Good Manufacturing Practice

GPC: Gel-Permeation Chromatography

HPLC: High Performance Liquid Chromatography

HILIC: Hydrophilic Interaction Chromatography

ICH: International Conference on Harmonization

IR: Infrared

KL: Kinapharma Ltd, Ghana

LPG: Letap Pharmaceuticals, Ghana

LPU: Luex Pharmaceuticals, UK

LOD: Limit of Detection

LOQ: Limit of Quantitation

NIR: Near-Infrared

NMR: Nuclear Magnetic Resonance

NSAIA: Nonsteroidal Anti-Inflammatory Agent

MGP: M & G Pharmaceticals Ltd, Ghana

MLL: Maxheal Laboratories Ltd, India

MS: Mass Spectrometry

ODS: Octadecylsilane

QbD: Quality by Design

QA: Quality Assurance

QC: Quality Control

Rf: Retardation factor

RSD: Relative Standard Deviation

SD: Standard Deviation

SEC: Size-Exclusion Chromatography

TLC: Thin Layer Chromatography

t_R: Retention Time

USP: United States Pharmacopoeia

UV-Vis: Ultra-Violet Visible

CHAPTER ONE

1.0 INTRODUCTION

During the past 30 years, high performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide. HPLC methods are useful in the determination of drugs in pharmaceutical dosage forms and biological samples. [1]

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals. [2,3]

HPLC-based techniques have long been a traditional mainstay of the pharmaceutical industry. It is a powerful technology that allows complex mixtures to be transformed into separated components. It is highly sensitive, reproducible, accessible, and well-understood from an operator's standpoint. Perhaps the output from the HPLC is its unique characteristic that distinguishes it from all other analytical techniques. The output from an HPLC, the chromatogram, is defined and simple. Each peak is characteristic of a component; each chromatogram is diagnostic of an event or experiment associated with a drug development activity. When combined with the fact that nearly all 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, it becomes evident why HPLC is a universally accepted analytical tool in the pharmaceutical industry. [4]

Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and that these are thoroughly validated. HPLC analytical method development is a critical process when using HPLC for pharmaceutical analysis. Methods need to separate the desired components satisfactorily, they need to generate the required results, and they must be reproducible and robust so they can be used time after time without problems.

The selection of suitable HPLC conditions to achieve a desired separation is often both confusing and time consuming. The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of the analytes will allow the most appropriate mode of HPLC to be selected. [5]

Since the mobile phase governs solute-stationary phase interactions, its choice is critical. Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided. Chemical purity of solvents is an important factor. Since large volume of solvent are pumped through the column, trace impurities can easily concentrate in the column and eventually be detrimental to the results. Spectrophotometric and HPLC grade solvents are recommended. Volatility should be considered if sample recovery is required. Viscosity should be less than 0.5 centipoise, otherwise high pump pressures are required and mass transfer between solvent and stationary phase will be reduced. [6]

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C8 phase (reverse phase) can provide a further time saving over a C18, as it does not retain analytes as strongly as the C18 phase. For normal phase applications, cyano (nitrile) phases are the most versatile. Method development can be streamlined by starting with shorter columns; 150, 100 or even 50 mm long. This is simply because they have proportionally shorter run times. [6]

The next consideration should be the choice of a detector. There is little use in running a separation if the detector one uses cannot detect all the components of interest, or conversely, if it detects too much. UV-vis detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity of some analyte. Unfortunately, UV–vis detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromophores, such as aromatic rings, for UV-vis detection.

There are three (3) critical components for a HPLC method, which are sample preparation, HPLC analysis and standardization (calculations). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization. [7]

The purpose of an internal standard in HPLC is to provide a reference concentration against which the responses of the target analytes are compared. By adding the internal standard to the sample just prior to instrumental analysis, the quantity present is not affected by extraction efficiencies or other sample handling procedures. The internal standard can then compensate for relatively minor fluctuations in instrument sensitivity to provide more accurate quantification of the target analytes. 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 surrogate standard is a compound that has properties similar to the target analyte(s) that a particular analytical method is designed to identify and measure. The surrogate compound is not expected to be in an environmental field sample and should not interfere with the identification

or quantification of the target analytes. By demonstrating that the surrogate compound can be recovered from the sample matrix with reasonable efficiency, the surrogate standard performs a quality control function on the suitability of the analytical method for the intended analyses and on the ability of the laboratory to execute that method with reasonable proficiency. If a surrogate compound is not recovered, an analyte of concern also may not be recovered. [8]

Proper validation of analytical methods is important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

Analytical method validation is now required by regulatory authorities for marketing authorizations and guidelines have been published. It is important to isolate analytical method validation from the selection and development of the method. Method selection is the first step in establishing an analytical method and consideration must be given to what is to be measured, and with what accuracy and precision.

Method development and validation can be simultaneous, but they are two different processes, both downstream of method selection. Analytical methods used in quality control should ensure an acceptable degree of confidence that results of the analyses of raw materials, excipients, intermediates, bulk products or finished products are viable. Before a test procedure is validated, the criteria to be used must be determined.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). The US Food and Drug Administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use.

The goals of HPLC method development have to be clearly defined, as pharmaceutical Quality by Design (QbD) is a systematic, scientific, riskbased, holistic and proactive approach that begins with predefined objectives and emphasizes product and process understanding and control. The ultimate goal of the analytical method is to separate and quantify the main compound and the critical quality attribute (CQA) impurities that may impact the quality of the drug product while meeting the method performance criteria based on regulatory requirements, such as specificity, linearity, accuracy, precision, sensitivity, robustness, and ruggedness. [9]

The use of HPLC has predominantly become the choice of analytical method in the pharmaceutical industry. Pure reference sample may not be available for all drugs and as such alternative methods need 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 reference powders of the target analytes, but chemically related compounds as surrogate reference standard.

1.1 JUSTIFICATION

Reference samples are an important tool in realizing a number of aspects of measurement of quality and are used for method validation, calibration, estimation of measurement uncertainty, training and for internal quality control (QC) and external quality assurance (QA) purposes. [10,11]

In quantitative analysis of drugs using HPLC, a pure reference standard is needed in the quality control monitoring of the drug. [7]

Generally the demand for reference materials exceeds supply in terms of the range of materials and availability. It is rare to have a choice of alternative reference samples and the user must choose the most suitable material available. It is important therefore that users and accreditation bodies understand any limitations of reference materials employed. [5]

The use of HPLC has predominantly become the choice of analytical method in the pharmaceutical industry. Pure reference sample may not be available for all drugs and as such alternative methods need 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 samples of the target analytes, but chemically related compounds analytes as surrogate reference standard.

In HPLC analysis of most drugs, a pure reference standard of the analyte is needed for quantitative analysis. There are times in industry and / or research when such standards are unavailable. In such situations, quality control monitoring of the drug both in-process and post

market becomes difficult for both industry and regulatory bodies to secure the ultimate interest of the consumer.

Reference standards from the United States of America are established and released with critical characteristics of each lot of specimen determined independently in three or more laboratories. The Food and Drugs Administration (FDA) laboratories and the United States Pharmacopoeia (USP) reference standards laboratory are involved in the testing of almost all new standards and replacements of existing standards [12]. Laboratories, both academia and industrial from all over the United States are also involved in the testing. Buying and importing these reference standards into Ghana however is expensive. The cost of Piroxicam and Metronidazole reference standards in August 2011 is given in Table 1.1.

A research to evolve a method that makes it possible to assess the quality of pharmaceuticals in the absence of the pure sample as a reference standard becomes imperative. A baseline study in this regard for Paracetamol, Aspirin, Diclofenac Sodium, Indomethacin, Diazepam and Prednisolone has successfully been completed at the Department of Pharmaceutical chemistry (KNUST). This project therefore seeks to extend the investigation by using the analysis of Piroxicam and Metronidazole as a test case.

Table 1.1 Cost of Piroxicam and Metronidazole pure reference samples

Catalog no	**Product description**	**Current lot**	**Previous lot**	**Unit price**
1544508	Piroxicam (200mg)	H2H258	H1D038(08/09)	$199.00 Each
1442009	Metronidazole (100mg)	J11272	J0C316 (08/11)	$199.00 Each

Source: USP Daily Reference Standards Catalog

1.2 MAIN OBJECTIVES

This project seeks to investigate the possibility of using surrogate reference standards for the analyses of Piroxicam capsules and Metronidazole tablets using HPLC.

1.2.1 SPECIFIC OBJECTIVES

The specific objectives of this research are;

- To develop an HPLC assay procedure for Piroxicam capsules using surrogate reference compounds.
- To develop an HPLC assay procedure for metronidazole tablets using surrogate reference compounds.
- To validate the method developed by using validation parameters such as Specificity and Selectivity, Linearity, precision, accuracy, Limit of detection (LOD), Limit of quantification (LOQ).
- To determine a constant, K that can effectively be used for quantitative analysis.
- To determine the percentage content of Piroxicam in various brand of capsules using the method developed.
- To determine the percentage content of Metronidazole in various brand of tablets using the method developed.
- To compare the results obtained from the method developed with a standard pharmacopeial method.

1.3 Hypothesis of study

For the same compounds,

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

For different compounds,

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

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

Therefore;

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

Hence

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

Where K is a constant analogous to the response factor of the solute

$A_{analyte}$ 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

Once K, $A_{analyte}$ and $C_{standard}$ are known for a particular system, $C_{analyte}$ can be calculated

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

Hence percentage content can be calculated as follows;

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

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Theory and Instrumentation of Various Analytical Techniques

2.1.1 High Performance Liquid Chromatography (HPLC)

2.1.1.1 History and general principles of HPLC

The emergence of liquid chromatography on a basis comparable with gas chromatography is usually considered to have started with a publication by Huber and Hulsman (1967). Although Giddings (1965) had already shown the potential advantages, in terms of column efficiencies and speed of analysis, two years earlier. The classic publication by Martin and Synge (1941), describing the development of partition chromatography, also made mention of the potential advantages of small particles and high pressures in liquid chromatography. A span of twenty six years elapsed, however before the major experimental advances, which brought about practical HPLC systems, were made by Horvath et a1 (1967).

Before this time, liquid chromatography had been performed in wide-bore glass columns, packed with large particle size stationary phases, operating at low solvent pressures with the eluent usually gravity-fed through the column. This resulted in both low efficiency separations and long analysis times, of up to several hours. The rapid development of gas chromatography (GC) in the 1960's also overshadowed liquid chromatography at this time, because of its high efficiency separations and relatively short analysis times. The development of liquid chromatographic columns of microparticulate silica (5-l0pm in diameter) and the use of high pressure eluent pumping systems resulted in the renaissance of liquid chromatography and the development of an analytical technique in which highly efficient separations could be achieved very rapidly on

involatile and thermally labile compounds and mixtures which in some cases could only be analysed by GC after prior derivatisation to more volatile components. [13]

2.1.1.2 Theory of liquid chromatography

Chromatography may be defined as the separation of the components of a mixture due to differences in the equilibrium distribution of the components between two phases, the mobile phase and the stationary phase. Components with a high distribution in the stationary phase will move more slowly through the column and thereby be separated from the components with a lower distribution in the stationary phase. [14, 15, 16]

2.1.1.3 Retention

The time elapsed between injection of a sample component onto an HPLC column and its subsequent detection is known as the retention time of that component (t_R). An unretained component passes through the column in a time t_o. The capacity factor of any component is a measure of its chromatographic retention. [14, 15, 16]

2.1.1.4 Column efficiency

This is a measure of the 'sharpness' of a chromatographic peak and has a direct bearing on the resolution of a chromatographic system. A number of band-broadening phenomena tend to reduce the efficiency of a chromatographic separation. These include: eddy diffusion, molecular diffusion, mass transfer and extra column effects. Connecting tubing between the column and detector may be one of the major factors in band broadening. It is necessary, therefore, to have short lengths of narrow-bore tubing, typically less than 0.25 mm internal diameter. The

maximum length of tubing between column injector and sample should not exceed 300 mm. [14, 15, 16]

2.1.1.5 Resolution

Substances are separated in a chromatographic column when their rates of migration differ. The ability of a stationary phase or solvent to produce the separation is a function of the thermodynamics of the system. The degree of resolution of two sample components is an experimentally derived parameter. [14, 15, 16]

2.1.1.6 Modes of chromatography

In the past the various different liquid chromatographic techniques have been regarded as separate and independent. Thus thin-layer chromatography (TLC) has largely employed liquid-solid adsorption as the basis of retention. Paper chromatography has been the main form of liquid-liquid partition chromatography, and ion-exchange resin beads have mainly been used for inorganic ions and amino acid analysis. The new equipment for HPLC has drawn these modes together by demonstrating that all have a common theoretical basis and can be carried out with the same basic HPLC equipment. The modes of chromatography differ only in 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. [17]

The majority of HPLC packing materials now in use are based upon wide-pore silica gels (i.e. silica gels with pores not less than 3 nm across). Such silica gels generally consist of fused aggregates of more or less spherical particles of colloidal silica.

The colloidal particles are of the order of 2-20 nm in diameter and the surface area is from 100-400 m^2 g^{-1}. The internal surface of a silica gel is covered with silanol groups (about four per nm^2) which can be used to adsorb polar solute molecules such as in adsorption chromatography, or be chemically modified to incorporate functional groups such as sulphonates, amines or alkyl groups. [17]

2.1.1.6.1 Adsorption chromatography

The basis of the separation achieved by adsorption chromatography is the selective adsorption of components of the mixture onto active sites on the surface of an adsorbent. Silica is the most widely used adsorbent. The active sites on silica are hydroxyl groups. Usually mobile phases such as hexane or dichloromethane are used to elute components from the column. [18]

2.1.1.6.2 Partition chromatography

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. Partition chromatography uses a retained solvent, on the surface or within the grains or fibres of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional coulombic and /or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent. Known as Hydrophilic Interaction Chromatography (HILIC) in HPLC, this method separates analytes based on polar differences. HILIC most often uses a bonded polar stationary phase and a non-polar, water miscible, mobile phase. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively

for separating analytes by relative polar differences, however, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram. [19]

The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. Retention strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends on the functional groups in the analyte molecule which promote partitioning but can also include coulombic (electrostatic) interaction and hydrogen donor capability. 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.

Partition and Normal Phase-HPLC had fallen out of favor in the 1970s with the development of reverse-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which improve reproducibility. [20]

2.1.1.6.3 Reverse Phase Chromatography

This technique is currently the most well known and most widely used liquid chromatographic mode in pharmaceutical analysis because of its ability to analyse both neutral and ionic compounds directly. The stationary phase is produced by reacting silica with materials such as

octadecyl trichlorosilane, to give, after hydrolysis and further treatment, a substituted surface with hydrocarbon characteristics. Other bondings include phenyl, octyl, cyano or amine functional groups as the hydrophobic surface layer.

Reverse phase columns can be used to retain ionic compounds by the use of pH control and/or the addition of modifiers to the mobile phase. By buffering the mobile phase in the pH range 2-5, weak acids can be retained. Similarly weak bases can be retained by ion suppression if pH control is in the range 7-8. Retention increases on the hydrophobic packing as ionisation is suppressed and a compound exists in its undissociated form. This technique is termed ion-suppression chromatography. Stronger acids and bases which are ionised in the pH range 2-8 can be separated by the technique of ion-pair chromatography. In this technique an appropriate counter ion is added to the mobile phase to pair with, and so increase retention of the ionic species. For acidic samples quaternary ammonium salts are commonly used as counter ions (at pH 7-8) and for basic samples, alkyl sulphonates are often used (at pH 3-4). [21]

2.1.1.6.4 Ion-exchange chromatography

Ion-exchange chromatography has a long history in biochemical and pharmaceutical applications. Typical ion-exchange materials contain either acidic groups such as sulphonic acid or carboxylic acid for the separation of cations or basic groups such as amine or quaternary ammonium salts for the separation of anions.

For separations based on polarity, like is attracted to like and opposites may be repelled. In ion-exchange chromatography and other separations based upon electrical charge, the rule is

reversed. Likes may repel, while opposites are attracted to each other. Stationary phases for ion-exchange separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain. Cation exchange is used to retain and separate positively charged ions on a negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface. With each type of ion exchange, there are at least two general approaches for separation and elution.

Strong ion exchangers bear functional groups e.g., quaternary amines or sulfonic acids, that are always ionized. They are typically used to retain and separate weak ions. These weak ions may be eluted by displacement with a mobile phase containing ions that are more strongly attracted to the stationary phase sites. Alternately, weak ions may be retained on the column, then neutralized by in situ changing the pH of the mobile phase, causing them to lose their attraction and elute.

Weak ion exchangers e.g., with secondary-amine or carboxylic-acid functions, may be neutralized above or below a certain pH value and lose their ability to retain ions by charge. When charged, they are used to retain and separate strong ions. If these ions cannot be eluted by displacement, then the stationary phase exchange sites may be neutralized, shutting off the ionic attraction, and permitting elution of the charged analytes.

When weak ion exchangers are neutralized, they may retain and separate species by hydrophobic (reverse-phase) or hydrophilic (normal-phase) interactions; in these cases, elution strength is determined by the polarity of the mobile phase. Thus, weak ion exchangers may be used for mixed-mode separations ie separations based on both polarity and charge. [22]

2.1.1.6.5 Size-Exclusion Chromatography or Gel-Permeation Chromatography

In the 1950s, Porath and Flodin discovered that biomolecules could be separated based on their size, rather than on their charge or polarity, by passing, or filtering, them through a controlled-porosity, hydrophilic dextran polymer. This process was termed gel filtration. Later, an analogous scheme was used to separate synthetic oligomers and polymers using organic-polymer packings with specific pore-size ranges. This process was called gel-permeation chromatography (GPC). Similar separations done using controlled-porosity silica packings were called size-exclusion chromatography (SEC). Introduced in 1963, the first commercial HPLC instruments were designed for GPC applications.

All of these techniques are typically done on stationary phases that have been synthesized with a pore-size distribution over a range that permits the analytes of interest to enter, or to be excluded from, more or less of the pore volume of the packing. Smaller molecules penetrate more of the pores on their passage through the bed. Larger molecules may only penetrate pores above a certain size so they spend less time in the bed. The biggest molecules may be totally excluded from pores and pass only between the particles, eluting very quickly in a small volume. Mobile phases are chosen for two reasons: first, they are good solvents for the analytes; and, second, they may prevent any interactions based on polarity or charge between the analytes and the stationary phase surface. In this way, the larger molecules elute first, while the smaller molecules travel slower because they move into and out of more of the pores and elute later, in decreasing order of their size in solution. Since it is possible to correlate the molecular weight of a polymer with its size in solution, GPC revolutionized measurement of the molecular-weight distribution

of polymers that, in turn, determines the physical characteristics that may enhance, or detract from, polymer processing, quality, and performance. [18]

2.1.1.7 Quantitative analysis in HPLC

In HPLC as in other chromatographic techniques, the peak area is proportional to the amount of compound eluted. Methods used for peak integration and for quantitative calculations correspond directly to those of GC. Integration algorithms, used in GC integrators or data-handling systems, can be applied directly for integration of HPLC peaks. For quantitative analysis the following instrumental requirements must be fulfilled: high flow stability, repeatable injection volumes, linearity of detector response and reliable integration parameters. Two methods of quantifying results are currently employed: the external standard and the internal standard method. In the former method absolute amounts are calculated from the ratio of sample peak areas to the corresponding standard peak areas obtained in the chromatogram. This method is mainly used for the higher sample concentrations found in such applications as pharmaceutical production control or formulation stability testing. The internal standard method is a very precise method for quantification. It reduces the possibility of error with injection reproducibility and with sample preparation procedures. This is especially important with analyses of low concentration samples found for example in pharmacokinetics and metabolism studies, where trace analysis of drugs or metabolites in a biological matrix is required. [23]

2.1.1.8 HPLC Instrumentation

Classical liquid chromatography used simple apparatus: a solvent reservoir, wide-bore glass columns, relatively large diameter particle packing and tubes for eluent collection. Detection was usually by chemical and physical methods and large samples were used. To increase the speed of

analysis and resolving power to match that of GC, it was necessary to increase the low liquid flow rates used, and also to obtain faster equilibration of the sample between the mobile and stationary phases so that resolution kept pace with faster flow rates. High performance liquid chromatography achieved these two aims by pressurising the mobile phase and by using packings of small particle size. This decreased the permeability of the column, so that to avoid excessively high pressures, shorter columns had to be used. The diameter of the column was also reduced since this increased efficiency and gave increased linear flow velocity for a given flow rate. Sample sizes also had to be reduced to prevent column overloading. A major breakthrough was the development of sensitive on-line detection systems capable of monitoring nanogram samples using small capacity flow cells to prevent loss of resolution. A modern liquid chromatograph then, consists basically of a high pressure pumping system, narrow bore short columns, packed with a small particle size stationary phase, and an on-line highly sensitive detection system. [24]

2.1.1.8.1 Mobile phase (solvent) reservoirs

For analytical separations the solvent reservoir should be ideally about 2 litres capacity, and is usually constructed from glass. Before solvent is used, however it should be degassed. Degassing is required to remove dissolved gases (in particular oxygen), which if not removed will produce degassing in the detector (as the solvent pressure is reduced from over 10^7 N m^{-2} to atmospheric pressure) which will produce either base-line drift or continuous spikes on the chromatographic trace. Before solvent is used it should also be filtered to remove any particulate matter which would cause wear to the pumping system. [24]

2.1.1.8.2 Pumping systems

The development of suitable pumping systems has been one of the main factors in the growth of modern liquid chromatography. Two types of elution are required, namely:

(i) isocratic, in which the solvent composition does not change during an analysis

(ii) gradient elution, during which continuous changes in composition are made at a controlled rate during the analysis.

Gradient elution is used in HPLC to separate the components of a mixture whose capacity factors (k') fall outside the range 1-10. In order for all components to be separated rapidly and efficiently some property of the solvent is programmed, e.g. mobile phase flow rate, polarity pH or ionic strength of the eluent, with respect to time. The basic requirements for a modern HPLC pumping system are as follows: maximum pressure range of about $3.5 \times 10^{7} Nm^{-2}$, pulse-free operation, flow delivery of between $0.1\text{-}10 mL min^{-1}$, with a reproducibility of ±0.5%. Conventional HPLC usually operates at about 1-2 mL min^{-1}. The pumping system must also be chemically inert to common solvents and a small volume (0.5 mL) pumping chamber is required for fast solvent changeover.

There are two basic types of pump in common use: constant pressure and constant volume pumps. Constant pressure pumps are of two main types: (i) coil pumps, in which inert gas at high pressure drives eluent out of a narrow tube, and (ii) air-driven pressure intensifiers (amplifiers) in which moderate air pressure drives a large area piston connected rigidly to a much smaller area piston bearing on the eluent. If constant volume pumps are used, changes in the pressure characteristics of the system, caused by settling or swelling of the packing, or viscosity changes

in the mobile phase caused either by temperature fluctuations or by composition changes manifest themselves as changes in the pressure rather than in the flow rate. Because flow changes cause non-reproducible retention times, adversely affect resolution and give unstable base lines, the constant volume pump provides a more precise analysis, particularly when gradient elution is employed.

Consequently constant pressure pumps have been superseded by constant volume pumps in virtually all modern HPLC systems. There are two main types of constant volume pump: constant displacement (syringe-type pumps) and reciprocating pumps - the latter being the most universally employed. In reciprocating pump, each forward stroke of the piston pushes solvent through the non-return valve (check valve) on the column side, and on the reverse stroke more solvent is drawn in from the solvent reservoir. Check valves at the inlet and outlet of the solvent chamber are an essential part of the pump design and must function well if the pump is to deliver an accurate flow of solvent. The main advantage of reciprocating pumps is that these internal volumes can be made very small and their delivery is continuous. Their main disadvantage is that solvent delivery is not pulse free particularly at high sensitivity settings. [25]

2.1.1.8.3 Sample introduction systems

For maximum efficiency on a chromatographic column, the sample should be introduced ideally in an infinitely narrow band. Valve-loop injectors are the most widely used sample introduction system and are designed to operate at pressures in excess of 3.5×10^7 N m^{-2}, without the use of septa. The solvent flow is bypassed into the column and an external loop is filled with sample,

which is then introduced into the column by switching a valve. The advantages of the loop-valve injector are:

(i) the ability to inject a wide range of sample volumes with a high degree of reproducibility.

(ii) the capability of injecting at high pressures (> 3.5 $x10^7$ N m^{-1}) without stopping the solvent flow,

(iii) the absence of a septum to cause column plugging,

(iv) the possibility of use with automatic sampling systems.

Their main disadvantage is that a reasonable volume of solute is required; it takes about l00µl to fill a l0µl sample loop properly.

Autosamplers are currently employed in most industrial analytical laboratories for rapid unattended analysis of samples. They can be used as stand-alone LC automation units or can become part of a remote controlled system. The sample injection valve is usually pneumatically activated, and provides excellent injector reproducibility. Since these systems can be operated 24 hours a day (with the aid of a time switch), the savings in time and manpower soon outweigh the cost of their installation. [25]

2.1.1.8.4 Columns and column fittings

The optimisation of columns and column fittings is particularly important when microparticulate packings (<10µm diameter) are being used. A typical column has dimensions of 250 mm x 4.6 mm and is constructed from stainless steel. Depending on the chromatographic mode required, the column is 'packed' under high pressure with suitable column packing, whose particles are typically less than 10µm in diameter. The method of packing usually employs balanced density

solvents and is a critical technical operation, which produces columns of high efficiency. For 3µm particles, efficiencies greater than 100 000 plates per metre have been obtained. To retain the packing in the column, porous plugs or frits are used. These are usually made from stainless steel and have a mesh size of 2-5µm. Control of column temperature is also important in HPLC and column thermostats are sometimes employed especially when using the reversed phase or ion exchange modes. [25]

2.1.1.8.5 Detectors

A major development in high efficiency HPLC has been the development of sensitive detection systems. Unlike GC, in liquid chromatography the physical properties of sample and mobile phase are often very similar. This has led to the development of two basic types of detector for use in HPLC.

(i) The differential measurement of a property common to both the sample and the mobile phase.

(ii) The measurement of a property that is specific to the sample, either with or without removal of the mobile phase before detection.

The characteristics of an ideal detector for HPLC include the following: high sensitivity, universal response to all solutes, or predicted specificity; linear response over several orders of concentration; low dead volume; non-destructive nature; insensitivity to temperature or flow changes; continuous operation; reliability and convenience of use.

Unfortunately no single detector satisfies all these criteria, but the detectors discussed in the following sections have found the most widespread use in HPLC. [26, 27, 28]

2.1.1.8.2 Types of HPLC Detectors

2.1.1.8.2.1 UV photometric detector

The most widely used HPLC detectors are based upon absorption of ultraviolet light. They are therefore not universal in application, but a great many substances, including most pharmaceuticals, do absorb UV radiation including all substances having π-bonding electrons and also those with unshared (non-bonded) electrons, such as olefins, aromatics and compounds containing carbonyl, thionyl, nitro and imine groups. The radiation source used in most uv detectors is a low pressure mercury vapour lamp. The predominant line in the spectrum is 254 nm and other lines may be filtered out to give monochromatic light at this wavelength.

Variable wavelength uv-visible spectrophotometers covering the range 190-800nm are most commonly employed as HPLC detectors.

The use of a photodiode array detector to capture uv-visible spectra during elution is a recent innovation. A 3D matrix of absorbance, wavelength and time data is generated, which can be interrogated in the wavelength domain, at a specified elution time, to give the conventional uv spectrum. Although uv spectra tend to be broad and relatively featureless, they can be used to aid in the identification of eluted solutes. The wavelength domain is added as a third dimension to time and absorbance, without stopping the eluent flow. This technique can be used in method development to characterise the components of an unknown mixture or to evaluate peak purity by absorbance ratioing methods. [29]

2.1.1.8.2.2 Refractive index detectors

These detectors continuously monitor the difference in refractive index between the pure mobile phase and the mobile phase plus eluted sample. The main advantage of these detectors is that

they are bulk property detectors and thus have universal application. To avoid drift, thermostatting of sample and reference cells to better than 0.001 ^{o}C is required. At its best the Refractive Index detector is about three orders of magnitude less sensitive than the uv detector. [30]

2.1.1.8.2.3 Fluorescence detectors

The number of compounds which fluoresce is smaller than those which show only absorption of light, thus fluorescence is a more specific technique. Furthermore it is also generally more sensitive. The solute is excited by UV radiation of a given wavelength and fluorescent energy is emitted at a longer wavelength. The emitted light is measured at right angles to the incident beam. [31]

2.1.1.8.2.4 Electrochemical detectors

Electrochemical detectors utilise the measurement of any electrical property to monitor a solute in an HPLC eluate. To date, capacitance, resistance: voltage and current have all been used and form the basis of permittivity, conductimetric, potentiometric and voltammetric detectors respectively. The latter detector is the most widely used electrochemical detector and can only be used for the detection of electroactive compounds. Two different approaches to voltammetry can be adopted:

(i) complete electrolysis of a solute (coulometry);

(ii) partial electrolysis of a solute (amperometry).

If amperometry is performed with mercury as the electrode material, this is known as polarography. [32]

2.1.1.8.2.5 Mass spectrometric detection

This is a very promising detector for HPLC. Most detectors have only a single parameter response so that the attributes of specificity and universal application are mutually exclusive. However with mass spectrometry it has a multi-parameter response and therefore it is possible for this system to be both specific and universal. The current interfaces can be classified into two basic types:

(i) belt transport interfaces which transfer the solute into the ion source after removal of solvent

(ii) direct liquid introduction (DLI), where the LC effluent is split and a small fraction is introduced into a chemical ionisation (CI) source. [33]

2.1.1.8.2.6 Miscellaneous detection systems

A large number of miscellaneous detection systems have also appeared in the literature during the last few decades. These include a Fourier transform infrared detector, an atomic absorption detector, an inductively-coupled plasma detector, a moving-wire flame ionisation detection system, a low angle laser light scattering detector, a radioactivity detector, a phosphorus-sensitive emission detector and many others. [33]

2.1.1.9 Applications of HPLC in pharmaceutical analysis

HPLC is widely used both in drug research and development and also pharmaceutical manufacture. Applications in research and development include purity control of new drug syntheses, separation of products during stability testing of drugs and formulations, and pharmacokinetics, i.e. the determination of drugs and metabolites in biological fluids during metabolism and clinical investigations. In pharmaceutical manufacture, HPLC is widely used in

the quality control of both raw materials and finished dosage forms, with pharmacopoeial monographs now containing several HPLC assay procedures. [34]

2.1.1.10 Applications in drug research and development

During drug development it is necessary to quantify drug stability in formulations and raw materials, with the identification of impurities and degradation products under a variety of environmental stress conditions. HPLC with its selectivity and sensitivity is ideally suited to such studies.

Drugs and formulated products can undergo degradation by, for example, oxidation, hydrolysis, photolysis, etc. It is then a requirement of pharmaceutical analysis to selectively determine each of these degradation products in the presence of the compound being analysed.

Amongst the classes of compound amenable to HPLC are alkaloids, antibiotics, nitrogenous and non-nitrogenous drugs, steroids, sulphur-containing compounds, vitamins, etc, covering the whole range of therapeutic activity. [34]

2.1.2 Thin Layer Chromatography

Thin layer chromatography (TLC) is among the most useful tools for following the progress of organic chemical reactions and for assaying the purity of organic compounds. TLC requires only a few nanograms (ng) of sample for a successful analysis and can be accomplished in a matter of minutes. Like all chromatographic methods, TLC takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve separation of complex mixtures of organic molecules. [35]

2.1.2.1 Theory of Thin Layer Chromatography

In thin layer chromatography, a solid phase, the adsorbent, is coated onto a solid support as a thin layer (about 0.25 mm thick). In many cases, a small amount of a binder such as plaster of Paris is mixed with the absorbent to facilitate the coating. Many different solid supports are employed, including thin sheets of glass, plastic, and aluminum. The mixture to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent, or mixture of solvents, called the eluant, is allowed to flow up the plate by capillary action. At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time sitting still on the adsorbent with the remainder moving up the plate with the solvent. A substance that is strongly adsorbed will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule that is strongly adsorbed will spend more time sitting still and less time moving. In contrast, a weakly adsorbed substance will have a smaller fraction of its molecules adsorbed at any one time, and hence any one molecule of the weakly adsorbed molecule will spend less time sitting and more time moving. Thus, the more weakly a substance is adsorbed, the farther up the plate it will move. The more strongly a substance is adsorbed, the closer it will stay near the origin.

Several factors determine the efficiency of a chromatographic separation. The adsorbent should show a maximum of selectivity toward the substances being separated so that the differences in rate of elution will be large. [35]

2.1.2.2 Technique of Thin-layer Chromatography

The sample is applied to the layer of adsorbent, near one edge, as a small spot of a solution. After the solvent has evaporated, the adsorbent-coated sheet is propped more or less vertically in a closed container, with the edge to which the spot was applied down. The spot on the thin layer plate must be positioned above the level of the solvent in the container. If it is below the level of the solvent, the spot will be washed off the plate into the developing solvent. The solvent, which is in the bottom of the container, creeps up the layer of adsorbent, passes over the spot, and, as it continues up, effects a separation of the materials in the spot and hence develops the chromatogram. When the solvent front has nearly reached nearly the top of the adsorbent, the thin layer plate is removed from the container. [36]

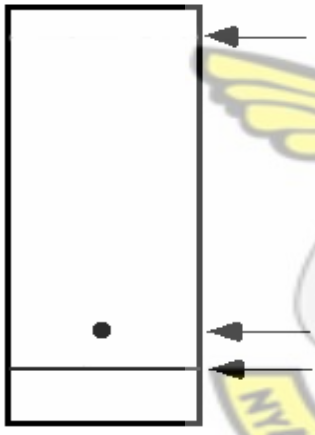

Figure 2.1 Position of the spot on a thin layer plate

Since the amount of adsorbent involved is relatively small, and the ratio of adsorbent to sample must be high, the amount of sample must be very small, usually much less than a milligram. For this reason, thin-layer chromatography (TLC) is usually used as an analytical technique rather than a preparative method. With thicker layers (about 2 mm) and large plates with a number of spots or a stripe of sample, it can be used as a preparative method. The separated substances are

recovered by scraping the adsorbent off the plate or cutting out the spots if the supporting material can be cut and extracting the substance from the adsorbent. [37]

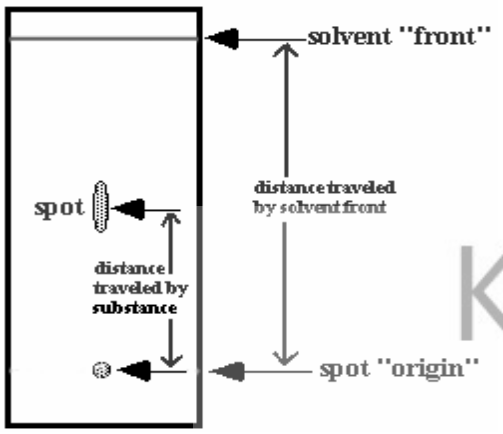

Figure 2.2 TLC plate showing distances travelled by the spot and the solvent after solvent front nearly reached the top of the adsorbent.

Because the distance travelled by a substance relative to the distance travelled by the solvent front depends upon the molecular structure of the substance, TLC can be used to identify substances as well as to separate them. The relationship between the distance traveled by the solvent front and the substance is usually expressed as the Rf value:

$$\text{Rf value} = \frac{\text{Distance travelled by substance}}{\text{(Distance travelled by solvent front)}}$$

The Rf values are strongly dependent upon the nature of the adsorbent and solvent. Therefore, experimental Rf values and literature values do not often agree very well. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances side by side in the same chromatogram, preferably at the same concentration. [38, 39]

2.1.2.3 Visualization

When the solvent front has moved to within about 1 cm of the top end of the adsorbent (after 15 to 45 minutes), the plate should be removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate. If the components of the sample are coloured, they can be observed directly. If not, they can sometimes be visualized by shining ultraviolet light on the plate or by allowing the plate to stand for a few minutes in a closed container in which the atmosphere is saturated with iodine vapour. Sometimes the spots can be visualized by spraying the plate with a reagent that will react with one or more of the components of the sample. [40, 41]

2.1.3 Ultraviolet-visible spectroscopy

Ultraviolet-visible spectroscopy (UV-Vis) refers to absorption spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived colour of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.[42]

UV/Vis spectroscopy is routinely used in the quantitative determination of solutions of transition metal ions and highly conjugated organic compounds.

Solutions of transition metal ions can be coloured (i.e., absorb visible light) because d electrons within the metal atoms can be excited from one electronic state to another. The colour of metal

ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulphate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λ_{max}).

Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water soluble compounds, or ethanol for organic-soluble compounds. Organic solvents may have significant UV absorption; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelengths. Solvent polarity and pH can affect the absorption spectrum of an organic compound. Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decreases. While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement. [43]

The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from references ie tables of molar extinction coefficients, or more accurately, determined from a calibration curve.

A UV/Vis spectrophotometer may be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. For accurate results, the instrument's response to the analyte in the unknown should be compared with the response to a

standard; this is very similar to the use of calibration curves. The response (e.g., peak height) for a particular concentration is known as the response factor. [44]

The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule. The Woodward-Fieser rules, for instance, are a set of empirical observations used to predict λ_{max}, the wavelength of the most intense UV/Vis absorption, for conjugated organic compounds such as dienes and ketones. The spectrum alone is not, however, a specific test for any given sample. The nature of the solvent, the pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances can influence the absorption spectrum. Experimental variations such as the slit width (effective bandwidth) of the spectrophotometer will also alter the spectrum. To apply UV/Vis spectroscopy to analysis, these variables must be controlled or accounted for in order to identify the substances present. [45]

UV/Vis spectroscopy is most often used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

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

Where A is the measured absorbance, I_0 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 absorbance and extinction ε are sometimes defined in terms of the natural logarithm instead of the base-10 logarithm.

The Beer-Lambert Law is useful for characterizing many compounds but does not hold as a universal relationship for the concentration and absorption of all substances. [46]

2.1.4 Nuclear Magnetic Resonance Spectroscopy

2.1.4.1 Background

Over the past fifty years, nuclear magnetic resonance spectroscopy, commonly referred to as NMR, has become the preeminent technique for determining the structure of organic compounds. Of all the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected. Although larger amounts of sample are needed than for mass spectroscopy, NMR is non-destructive, and with modern instruments good data may be obtained from samples weighing less than a milligram.

Many types of information can be obtained from an NMR spectrum. Much like using infrared spectroscopy (IR) to identify functional groups, analysis of a NMR spectrum provides information on the number and type of chemical entities in a molecule. However, NMR provides much more information than IR.

The impact of NMR spectroscopy on the natural sciences has been substantial. It can, among other things, be used to study mixtures of analytes, to understand dynamic effects such as change in temperature and reaction mechanisms, and is an invaluable tool in understanding protein and nucleic acid structure and function. It can be applied to a wide variety of samples, both in the solution and the solid state.

2.1.4.2 Basic NMR techniques

The NMR sample is prepared in a thin-walled glass tube - an NMR tube. When placed in a magnetic field, NMR active nuclei (such as ^{1}H or ^{13}C) absorb at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. For example, in a 21 tesla magnetic field, protons resonate at 900 MHz. It is common to refer to a 21 T magnet as a 900 MHz magnet, although different nuclei resonate at a different frequency at this field strength.

In the Earth's magnetic field the same nuclei resonate at audio frequencies. This effect is used in Earth's field NMR spectrometers and other instruments. Because these instruments are portable and inexpensive, they are often used for teaching and field work. [47]

2.1.4.3 Chemical Shift

Unlike infrared and uv-visible spectroscopy, where absorption peaks are uniquely located by a frequency or wavelength, the location of different NMR resonance signals is dependent on both the external magnetic field strength and the radio frequency. Since no two magnets will have exactly the same field, resonance frequencies will vary accordingly and an alternative method for characterizing and specifying the location of NMR signals is needed. [48]

2.1.4.4 Signal Strength

The magnitude or intensity of NMR resonance signals is displayed along the vertical axis of a spectrum, and is proportional to the molar concentration of the sample. Thus, a small or dilute sample will give a weak signal, and doubling or tripling the sample concentration increases the

signal strength proportionally. Considering the NMR spectrum of equal molar amounts of benzene and cyclohexane in carbon tetrachloride solution, the resonance signal from cyclohexane will be twice as intense as that from benzene because cyclohexane has twice as many hydrogens per molecule. This is an important relationship when samples incorporating two or more different sets of hydrogen atoms are examined, since it allows the ratio of hydrogen atoms in each distinct set to be determined. To this end it is necessary to measure the relative strength as well as the chemical shift of the resonance signals that comprise an NMR spectrum. [48]

2.1.5 Infrared Spectroscopy

Infrared (IR) spectroscopy is one of the most common spectroscopic techniques used by organic and inorganic chemists. Simply, it is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids, and solids. Thus, IR spectroscopy is an important and popular tool for structural elucidation and compound identification. [49]

Infrared radiation spans a section of the electromagnetic spectrum having wavenumbers from roughly 13,000 to 10 cm^{-1}, or wavelengths from 0.78 to 1000 μm. It is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies. IR absorption positions are generally presented as either wavenumbers ($\bar{\upsilon}$) or wavelengths (l).

Wavenumber defines the number of waves per unit length. Thus, wavenumbers are directly proportional to frequency, as well as the energy of the IR absorption. The wavenumber unit (cm^{-1}, reciprocal centimeter) is more commonly used in modern IR instruments that are linear in the cm^{-1} scale. In the contrast, wavelengths are inversely proportional to frequencies and their associated energy. At present, the recommended unit of wavelength is μm (micrometers).

IR absorption information is generally presented in the form of a spectrum with wavelength or wavenumber as the x-axis and absorption intensity or percent transmittance as the y-axis

Transmittance, *T*, is the ratio of radiant power transmitted by the sample (*I*) to the radiant power incident on the sample (I_0). Absorbance (*A*) is the logarithm to the base 10 of the reciprocal of the transmittance (*T*). The transmittance spectra provide better contrast between intensities of strong and weak bands because transmittance ranges from 0 to 100% *T* whereas absorbance ranges from infinity to zero. [50]

The IR region is commonly divided into three smaller areas: near IR, mid IR, and far IR. The most frequently used is the mid IR region, between 4000 and 400 cm−1 (2.5 to 25 μm). The far IR requires the use of specialized optical materials and sources. It is used for analysis of organic, inorganic, and organometallic compounds involving heavy atoms (mass number over 19). It provides useful information to structural studies such as conformation and lattice dynamics of samples. Near IR spectroscopy needs minimal or no sample preparation. It offers high-speed quantitative analysis without consumption or destruction of the sample. Its instruments can often be combined with UV-visible spectrometer and coupled with fiberoptic devices for remote

analysis. Near IR spectroscopy has gained increased interest, especially in process control applications. [51, 52]

2.1.5.1 Analytical Information Applications

2.1.5.1.1 Qualitative

The combination of the fundamental vibrations or rotations of various functional groups and the subtle interactions of these functional groups with other atoms of the molecule results in the unique, generally complex IR spectrum for each individual compound. IR spectroscopy is mainly used in two ways: structural elucidation and compound identification. [53]

Structural Elucidation

Because of complex interactions of atoms within the molecule, IR absorption of the functional groups may vary over a wide range. However, it has been found that many functional groups give characteristic IR absorption at specific, narrow frequency ranges regardless of their relationship with the rest of the molecule. Multiple functional groups may absorb at one particular frequency range, but a functional group often gives rise to multiple-characteristic absorption. Thus, the spectral interpretations should not be confined to one or two bands and the whole spectrum should be examined. To confirm or better elucidate the structure of an unknown substance, other analytical information provided by nuclear magnetic resonance (NMR), mass spectrometry (MS), or other chemical analysis should also be used where possible. [54]

Compound Identification

Since the IR spectrum of every molecule is unique, one of the most positive identification methods of an organic compound is to find a reference IR spectrum that matches that of the unknown compound. A large number of reference spectra for vapour and condensed phases are available in printed and electronic formats. The spectral libraries compiled by Sadtler and Aldrich are some of the most popular collections. In addition, spectral databases are often compiled according to application areas such as forensics, biochemicals, and polymers. Computerized search programs can facilitate the matching process. In many cases where exact match to the spectrum of an unknown material cannot be found, these programs usually list the reference compounds that match the unknown spectrum most closely. This information is useful in narrowing the search. When it is combined with the data from other analysis such as NMR or mass spectrometry, a positive identification or high-confidence level tentative identification can often be achieved. [55]

2.1.5.1.2 Quantitative

IR spectroscopy was generally considered to be able to provide only qualitative and semiquantitative analyses of common samples, especially when the data were acquired using the conventional dispersive instruments. However, the development of reliable FTIR instrumentation and strong computerized data-processing capabilities has greatly improved the performance of quantitative IR work. Thus, modern infrared spectroscopy has gained acceptance as a reliable tool for quantitative analysis. The basis for quantitative analysis of absorption spectrometry is the Bouguer–Beer–Lambert law, commonly called Beer's law. [56]

2.2. Introduction to analytes and surrogate reference standards

2.2.1 Piroxicam

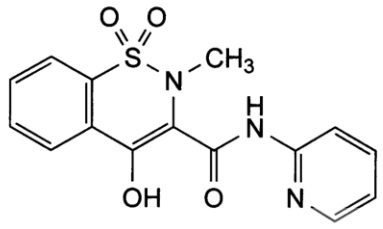

Figure 2.3 Chemical structure of Piroxicam $C_{15}H_{13}N_3O_4S$ [57]

Piroxicam, 4-hydroxy -2- methyl –*N* -(pyridin-2-yl) -2*H*-1,2- benzothiazine - 3 -carboxamide 1,1 dioxide[57] an oxicam derivative, is a prototypical nonsteroidal anti-inflammatory agent (NSAIA). This drug has been widely used in the treatment of rheumatoid arthritis and other inflammatory disorders. [58]

Piroxicam occurs as a white, crystalline solid and is sparingly soluble in water and slightly soluble in alcohol and in alkaline aqueous solution.[57] Piroxicam has a pK_a of 5.1 in aqueous solution.

Piroxicam is structurally unrelated to other NSAIAs. The drug is acidic because of the presence of a 4-hydroxy enolic acid substituent.

Piroxicam capsules should be stored in tight, light-resistant containers at a temperature less than 30°C. When stored under recommended conditions, piroxicam capsules are stable for 36 months after the date of manufacture. [58]

The usual dose is 20 mg by mouth. It is well absorbed from the gastrointestinal tract, is metabolized in the liver by hydroxylation and conjugation with glucuronic acid, and is excreted predominantly in the urine with smaller amounts in the feaces. Less than 5% of the dose is excreted unchanged. [59, 60]

Piroxicam is extensively (99%) bound to plasma proteins and has a long plasma half-life of approximately 50 hours. [59, 60]

2.2.2 Metronidazole

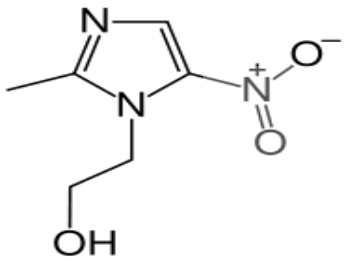

Figure 2.4 Chemical structure of Metronidazole $C_6H_9N_3O_3$ [57]

Metronidazole 2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol[57] commonly referred to as Flagyl is a nitroimidazole antibiotic drug. Nitroimidazoles are imidazole heterocycles that have a nitro group that is used to combat anaerobic bacterial and parasitic infections. The group was originally introduced in 1957 to treat vaginal infections by amoeba, but is now used for the treatment of pseudomembranous enterocolitis caused by Clostridium difficile, an opportunistic pathogen, and as part of a cocktail for Helicobactor pylori. [61]

It appears as odourless crystals with colour ranging from white to pale yellow. It darkens when exposed to light but it is stable in air and has a melting point between 159°C and 163°C. It is sparingly soluble in water, alcohol and chloroform and slightly soluble in ether.[57] Metronidazole is a weakly basic drug. It acts as a Lewis base when the lone-pair of electrons on Nitrogen is donated during a reaction. [61]

Metronidazole is also used to treat giardia infections of the small intestines, amoebic liver abscess and dysentery, trichomonas vaginal infections, and carriers of trichomonas (both sexual partners) who do not have symptoms of infection. Metronidazole is also used alone or in combination with other antibiotics in treating abscesses in the liver, pelvis, abdomen and brain caused by susceptible anaerobic bacteria. The drug diffuses into the cells of protozoa or anaerobic bacteria, where the nitro group is reduced by a "ferredoxin-mediated electron transport system," producing short-lived toxic radicals. The formation of the toxic radical also helps more Metronidazole diffuse into the cell, since the intracellular concentration of Metronidazole decreases as more prodrug is created. [62]

2.2.3 Ascorbic acid

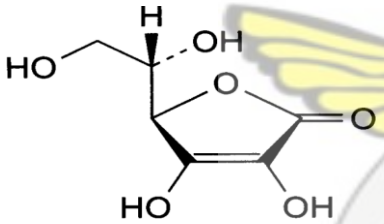

Figure 2.5 Chemical structure of ascorbic acid $C_6H_8O_6$ [57]

Ascorbic acid (5*R*)-5-[(1*S*)-1, 2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one[57] is a sugar acid with antioxidant properties. One form of ascorbic acid is commonly known as vitamin C. It is a coenzyme in tyrosine oxidation.[63] It creates volatile compounds when mixed with glucose and amino acids.[64]

It appears as a white or almost white, crystalline powder or colourless crystals, becoming discoloured on exposure to air and moisture, freely soluble in water, soluble in alcohol, practically insoluble in ether. It melts at about 190 °C, with decomposition. [57]

Ascorbic acid behaves as a vinylogous carboxylic acid where the electrons in the double bond, hydroxyl group lone pair, and the carbonyl double bond form a conjugated system. Because the two major resonance structures stabilize the deprotonated conjugate base of ascorbic acid, the hydroxyl group in ascorbic acid is much more acidic than typical hydroxyl groups. In other words, ascorbic acid can be considered an enol where the deprotonated form is a stabilized enolate. [65]

Ascorbic acid also interconverts into two unstable ketone tautomers by proton transfer, although it is the most stable in the enol form. The proton of the hydroxyl of the enol is removed. Then a pair of electrons from the resulting oxide anion pushes down to form the ketone at the 2 or 3 position and the electrons from the double bond move to the 3 or 2 position, respectively, forming the carbanion, which picks up the proton resulting in two possible forms: 1-carboxyl-2-ketone and 1-carboxyl-3-ketone.

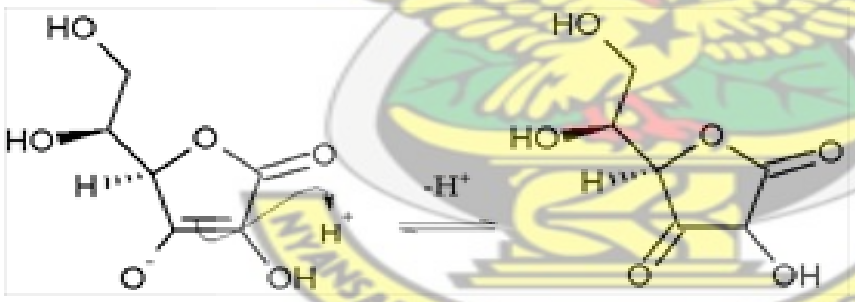

Fig 2.6 Formation of the unstable ketone tautomer by proton transfer

The concentration of a solution of ascorbic acid can be determined in many ways, the most common ways involving titration with an oxidizing agent such as iodine. Another method involves using iodine and a starch indicator, wherein iodine reacts with ascorbic acid, and, when all the ascorbic acid has reacted, the iodine is then in excess, forming a blue-black complex with the starch indicator. This indicates the end-point of the titration. As an alternative, ascorbic acid

can be treated with iodine in excess, followed by back titration with sodium thiosulfate using starch as an indicator. [66]

2.2.4 Para aminophenol

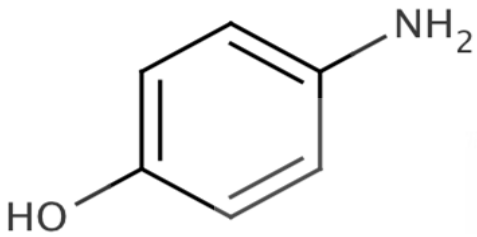

Figure 2.7 Chemical structure of Para aminophenol C_6H_7NO [57]

Para aminophenol (4 Aminophenol) is the organic compound typically available as a white powder. It is commonly used as a developer in black-and-white film, marketed under the name Rodinal. [67]

Para aminophenol appears as a white or slightly coloured, crystalline powder with melting point of about 186°, with decomposition. [57] Reflecting its slight hydrophilic character, the white powder is moderately soluble in alcohols and can be recrystallised from hot water. In the presence of base, it oxidizes readily. The *N*-methyl and *N*,*N*-dimethyl derivatives are of commercial value.

The compound is one of three isomeric aminophenols, the other two being 2-aminophenol and 3-aminophenol. It is produced from phenol by nitration followed by reduction with iron. Alternatively, the partial hydrogenation of nitrobenzene affords phenylhydroxylamine, which rearranges primarily to 4-aminophenol:[68]

$C_6H_5NO_2 + 2\ H_2 \rightarrow C_6H_5NHOH + H_2O$

$C_6H_5NHOH \rightarrow HOC_6H_4NH_2$

Para aminophenol reacts with acetic anhydride to give paracetamol.[69]

4-aminophenol is considered a minor nephrotoxic metabolite of phenacetin and acetaminophen (paracetamol) in man. 4-Aminophenol can undergo autoxidations and metal-catalyzed and enzymatic oxidations in man to produce reactive oxygen species. [70]

2.2.5 Metformin Hydrochloride

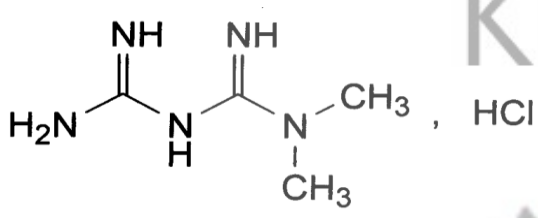

Figure 2.8 Chemical structure of metformin hydrochloride $C_4H_{12}ClN_5$ [57]

Metformin hydrochloride, 1,1-Dimethylbiguanide hydrochloride[57] originally sold as Glucophage is an oral anti-diabetic drug in the biguanide class. It is the first-line drug of choice for the treatment of type 2 diabetes, particularly in overweight and obese people and those with normal kidney function. Evidence is also mounting for its efficacy in gestational diabetes, although safety concerns still preclude its widespread use in this setting. It is also used in the treatment of polycystic ovary syndrome and has been investigated for other diseases where insulin resistance may be an important factor.

It appears as white crystals and it is freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone and in methylene chloride. Its melting point ranges from 222 °C to 226 °C. [57]

Metformin helps reduce Low-density Lipoprotein (LDL) cholesterol and triglyceride levels and is not associated with weight gain, and is the only anti-diabetic drug that has been conclusively shown to prevent the cardiovascular complications of diabetes. Metformin is now believed to be the most widely prescribed anti-diabetic drug in the world; in the United States alone, more than 42 million prescriptions were filled in 2009 for its generic formulations.[71][72]

The biguanide class of anti-diabetic drugs, which also includes the withdrawn agents phenformin and buformin, originates from the French lilac (*Galega officinalis*), a plant used in folk medicine for several centuries.[73]

The usual synthesis of metformin, originally described in 1922 and reproduced in multiple later patents and publications, involves the reaction of dimethylamine hydrochloride and 2-cyanoguanidine (dicyandiamide) with heating.[74][75]

The structure of metformin was generally represented in a wrong tautomeric form for several years. This was corrected in 2005.[76] The energy difference between the correct tautomer and the generally represented tautomer is about 9 kcal/mol. The drug is administered as metformin hydrochloride.[77][78]

CHAPTER THREE

3.0 EXPERIMENTAL METHODS, MATERIALS AND REAGENTS

3.1 Materials / Reagents

Perchloric acid, 70% A.C.S (Aldrich), Anhydrous acetic acid (BDH), Acetic anhydride (BDH), Methanol (HPLC grade), Potassium dihydrogen orthophosphate (BDH), Hydrochloric acid, 32% (BDH), Sodium hydroxide pellets 99% (BDH), Anhydrous formic acid (BDH), Acetonitrile (BDH), Starch solution, Iodine, Sodium thiosulphate (BDH), Potassium hydrogen phthalate (BDH)

These reagents were provided by the Department of Pharmaceutical Chemistry, KNUST-Kumasi, Ghana

Piroxicam pure sample was obtained from Ernest Chemist Limited while Metronidazole, Metformin and Ascorbic acid pure samples were obtained from Amponsah Effah Pharmaceuticals for this project.

Table 3.1 Information on pure samples used

Name	**Batch no**	**Date of manufacture**	**Date of expiry**	**Percentage purity**
Piroxicam	B/N K8-19	4/10	3/12	100.08
Metronidazole	09011801	09/11/09	12/09/2012	99.80
Metformin	Q137	28/09/2010	31/02/2012	99.50
Ascorbic acid	001/847	20/6/2009	19/6/2012	99.27

Para aminophenol was obtained from the Pharmaceutical Chemistry Department at KNUST, Kumasi, Ghana.

Four brands each of Piroxicam capsules and Metronidazole tablets manufactured by different manufacturing companies were used in this project. The brands were obtained from different Pharmacy shops in Bantama, Kumasi. The description of each brand is as follows;

Table 3.2 Brands of Piroxicam

Capsule	**Cap. strength (mg)**	**Name of manufacturer**	**Batch no.**	**Date of manufacture**	**Date of expiry**
Piroxicam ECL	20	Ernest Chemist Ltd, Ghana	0508J	Aug. 2009	Aug. 2013
Piroxicam KL	20	Kinapharma Ltd, Ghana	10012	Nov. 2010	Nov. 2013
Piroxicam LPG	20	Letap Pharmaceuticals, Ghana	0210131	March 2011	Feb. 2013
Piroxicam LPU	20	Luex Pharmaceuticals, UK	100428	April 2010	March 2013

Table 3.3 Brands of Metronidazole

Tablet	**Tablet strength (mg)**	**Name of manufacturer**	**Batch no.**	**Date of manufacture**	**Date of expiry**
Metronidazole ECL	200	Ernest Chemist Ltd, Ghana	0701L	June 2010	June 2015
Metronidazole LPG	200	Letap Pharmaceuticals, Ghana	0740881	Feb. 2010	March 2013
Metronidazole MGP	200	M & G Pharmaceticals Ltd, Ghana	MZ119	Feb. 2010	Feb. 2014
Metronidazole MLL	200	Maxheal Laboratories Ltd, India	XT015	June 2010	May 2013

3.2 Instrumentation

An HPLC set up consisting of the following were used;

- Shimadzu LC-10AS Liquid Chromatograph Pump
- Kontron Instrument HPLC Pump 422
- ODS C 18 Phenomenex 250 x 4.6mm column
- Applied Biosystems 783A Programmable Absorbance Detector
- eDAQ Power Chrom Software 280
- HP Computer work station

Other equipment used include the following

- Whatman Filter paper 11.0 cm
- Adam PW / 24 Analytical weighing balance
- Cecil CE 2041 2000 Series-UV Spectrophotometer
- Eutech Instrument pH 510/ pHmV/ ^{o}C Meter
- Chromato-Vue C-70 UV View System (UVP inc) 254nm Short wave; 365nm Long wave; White light
- Stuart Melting Point SMP 10 Apparatus
- Melting point capillary tubes
- Sonicator

3.3 Identification tests for samples

3.3.1 Qualitative tests (British Pharmacopoeia 2007)

3.3.1.1 Metronidazole pure sample

40.0 mg of metronidazole pure powder was dissolved in 0.1 M hydrochloric acid and diluted to 100.0 mL with the same acid. 5.0 mL of the solution was diluted to 100.0 mL with 0.1M hydrochloric acid. The resulting solution was examined between 230 nm and 350 nm. The specific absorbance at the maximum was then determined.

3.3.1.2 Ascorbic acid pure sample

1. 0.10 g of ascorbic acid pure powder was dissolved in distilled water and diluted immediately to 100.0 mL with the same solvent. 10 mL of 0.1 M hydrochloric acid was added to 1.0 mL of the solution and diluted to 100.0 mL with distilled water. The absorbance was measured at the maximum at 243 nm immediately after dissolution. The specific absorbance at the maximum was then determined.

2. 1.0 g of ascorbic acid pure powder was dissolved in carbon dioxide-free water and diluted to 20 mL with the same solvent. The pH of resulting solution was determined.

3. 1.0 g of ascorbic acid pure powder was dissolved in carbon dioxide-free water and diluted to 20 mL with the same solvent. 1 mL of solution was taken and 0.2 mL of dilute nitric acid added. 0.2 mL of silver nitrate solution was then added to the resulting solution. A visual observation of the resulting solution was made.

3.3.1.3 Melting Point Determination

The dry pure powders of each of the pure samples were introduced into separate capillary tubes sealed at one end. The solid was shaken down the tube by tapping the sealed end on a hard surface so as to form a tightly packed column from 3 to 5 mm in height. These were then placed in a melting point determination apparatus and their various melting points determined.

3.3.2 Assay of pure samples (British Pharmacopoeia 2007)

3.3.2.1 Piroxicam

0.2503 g of piroxicam pure sample was dissolved in 60 mL of a mixture of equal volumes of acetic anhydride and anhydrous acetic acid. The resulting solution was then titrated with 0.1 M perchloric acid. The end-point was determined potentiometrically.

1 mL of 0.1 M perchloric acid is equivalent to 33.14 mg of $C_{15}H_{13}N_3O_4S$.

3.3.2.2 Metronidazole

0.1502 g of metronidazole pure sample was dissolved in 50 mL of anhydrous acetic acid and titrated with 0.1 M perchloric acid. The end-point was determined potentiometrically.

1 mL of 0.1 M perchloric acid is equivalent to 17.12 mg of $C_6H_9N_3O_3$.

3.3.2.3 Metformin

0.1005 g of metformin pure sample was dissolved in 4 mL of anhydrous formic acid. 80 mL of acetonitrile was then added. Titration was carried out immediately with 0.1 M perchloric acid, determining the end-point potentiometrically.

1 mL of 0.1 M perchloric acid is equivalent to 16.56 mg of $C_4H_{12}ClN_5$.

3.3.2.4 Ascorbic acid

0.1504 g ascorbic acid pure sample was dissolved in a mixture of 10 mL of 1M sulphuric acid and 80 mL of carbon dioxide-free water. 1 mL of starch solution was added. The resulting solution was then titrated with 0.05 M iodine until a persistent violet-blue colour was obtained.

1 mL of 0.05 M iodine is equivalent to 8.81 mg of $C_6H_8O_6$.

3.3.3 Thin Layer Chromatography (TLC)

3.3.3.1 Piroxicam pure sample and capsules (United States Pharmacopoeia 2007)

A quantity of the powdered Piroxicam capsules containing 10mg of Piroxicam was dissolved in a mixture of chloroform and methanol (1:1) and made up to 10mL with the same solvent. 10mg of pure piroxicam powder was also dissolved in the same solvent and made up to 10mL.

The chromatography was carried out using a Thin Layer Chromatographic plate coated with a 0.25mm layer of chromatographic silica gel as the stationary phase and a mobile phase composition of toluene and glacial acetic acid (95:5).

20µl portions of the test solution and the standard solution were spotted separately on the TLC plate and allowed to dry. The chromatogram was developed in the developing chamber containing the mobile phase until the solvent front has moved about three-fourths of the length of the plate

The plate was then removed and air dried and then observed under UV light at a wavelength of 254nm.

The Rf values of both the standard and sample were then compared.

3.3.3.2 Metronidazole pure sample and tablets (Food and Drugs Board, Ghana)

A quantity of the powdered Metronidazole tablets containing 200mg of Metronidazole was dissolved in 10mL methanol. 1mL of the stock solution was pipette and diluted with 3mL of methanol. A standard solution of pure Metronidazole powder was also prepared as above.

The chromatography was carried out using a Thin Layer Chromatographic plate coated with a 0.25mm layer of chromatographic silica gel as the stationary phase and a mobile phase composition of 15mL ethyl acetate, 5mL methanol and 10 drops of concentrated Ammonia solution.

20µl portions of the test solution and the standard solution were spotted separately on the TLC plate and allowed to dry. The chromatogram was developed in the developing chamber containing the mobile phase until the solvent front has moved about three-fourths of the length of the plate

The plate was then removed and air dried and then observed under UV light at a wavelength of 254nm.

The Rf values of both the standard and sample were then compared.

3.4 Standardization of Solutions

3.4.1 Standardization of 0.1M Perchloric Acid

Potassium hydrogen phthalate (0.5006g) was weighed into a 100mL conical flask. Glacial acetic acid (25mL) was added. It was then warmed for the dissolution of the salt.

Subsequently, it was cooled and titrated with Perchloric acid (0.1M). Oracet blue was used as the indicator

3.4.2 Standardization of 0.05M Iodine solution using Sodium Thiosulphate

25mL of the 0.05M iodine was pipetted into a conical flask. It was then titrated with the 0.1M Sodium thiosulphate from the burette until the iodine is decolourised at the endpoint.

3.5 Uniformity of weight

3.5.1 Uniformity of weight of Piroxicam capsules (British Pharmacopoeia 2007)

An intact capsule was weighed. The capsule was then opened without losing any part of the shell and the contents removed as completely as possible. The shell was then weighed. The difference between the weighings represents the mass of the content. The procedure was repeated with another 19 capsules. The average weight was determined for each of the brands. The deviation and percentage deviation were calculated for each of the weights taken.

3.5.2 Uniformity of weight of Metronidazole tablets (British Pharmacopoeia 2007)

20 tablets each of the four brands of metronidazole tablets were weighed individually and recorded. The average weight was determined for each of the brands. The deviation and percentage deviation were calculated for each of the weights taken.

3.6 Determination of Percentage content of Piroxicam in Piroxicam capsules (20mg) using Standard Method in the British Pharmacopoeia (2007)

A quantity of the mixed contents of 20 capsules containing 10 mg of Piroxicam was added to 150 mL of 0.01M methanolic hydrochloric acid and mixed with the aid of a sonicator for 30 minutes. The resulting solution was then cooled and diluted to 200 mL with the same solvent and

filtered through a glass-fibre paper. A solution containing 0.005% w/v of piroxicam pure sample in 0.01M methanolic hydrochloric acid was prepared as the standard solution.

The chromatographic procedure was carried out using a stainless steel octadecylsilyl column (30 cm × 3.9 mm), a flow rate of 2mL per minute and a mobile phase mixture of 60 volumes of methanol and 40 volumes of a buffer solution prepared by adding a solution containing 5.35 g of disodium hydrogen orthophosphate in 100 mL of water to a solution containing 7.72 g of citric acid in 400 mL of water and diluted to 1000 mL and a detection wavelength of 242 nm.

Equal volumes of the standard preparation and the assay preparation were injected separately into the chromatograph. The chromatogram was recorded and their respective peak responses noted.

The content $C_{15}H_{13}N_3O_4S$ was calculated using the declared content of $C_{15}H_{13}N_3O_4S$ in piroxicam BP.

3.7 Determination of Percentage content of Metronidazole in Metronidazole tablets (200mg) using Standard Method in the United States Pharmacopoeia (2007)

20 tablets of Metronidazole were weighed accurately and powdered. A quantity of the powder equivalent to 100mg was weighed and transferred into a 10mL volumetric flask. A quantity of the methanol was added and shaken by mechanical means for 30 minutes until all the powder has dissolved. Methanol was then added to the mark and filtered.

5mL of the filtrate was then pipetted into a 100mL volumetric flask and diluted with the mobile phase to volume and mixed. The solution was then filtered.

The reference standard was prepared by weighing a quantity of USP metronidazole pure sample in the mobile phase to obtain a solution having a known concentration of about 0.5mg per mL.

The chromatographic procedure was carried out using a stainless steel octadecylsilyl column (15cm × 4.6 mm) with a flow rate of 1 mL per minute, a mobile phase mixture of 80 volumes of water and 20 volumes of methanol and a detection wavelength of 254 nm.

Equal volumes of the standard preparation and the assay preparation were injected separately into the chromatograph. The chromatogram was recorded and their respective peak responses noted.

The quantity of metronidazole in the tablet was then calculated.

3.8 HPLC Method Development

3.8.1 Chromatographic conditions

When developing a rugged method, it is desirable to select a mobile phase with a final pH at least one pH unit away from any analyte's pK value to cause ionization or suppression of the analytes.

Slight variations in mobile phase preparation can result in pH changes that can have dramatic effects on selectivity, capacity factor (retention factor), peak shape, resolution, and reproducibility. Optimum pH control will usually result in mobile phase containing buffer and acid compositions that will resist change when the sample is introduced and force ionizable analytes into predominantly one form (ionized or neutral) as they enter the column.

Mobile phase pH should be selected so that it is at least ± 1.5 pH units from the analyte's pKa. This assures that the analytes are either 100% ionized or 100% non-ionized and should help control run-run reproducibility. At high pH, acidic compounds are ionized and are much more hydrophilic than under ion suppression conditions. These conditions should be selected when fast analysis and low retention are desired.

To obtain chromatograms with a good separation and resolution of adjacent peaks within a short analysis time, the mobile phase, UV absorption and flow rate were optimized.

3.8.2 Chromatographic system for Piroxicam and its surrogates

During analytical method development, the mixtures of methanol and KH_2PO_4 in different combinations at different pH were investigated at different flow rates to determine the effect on elution of piroxicam and the surrogate reference standards under isocratic conditions. The best separation was obtained by use of methanol- phosphate buffer pH 6.2 in the ratio 50:50, as mobile phase at a flow rate of 1mL/min, because under these conditions the chromatographic peaks were better defined, better resolved and free from tailing.

A reverse phase HPLC would be performed hence the choice of an ODS C 18 Phenomenex 250 x 4.6mm column 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, 254nm was the optimum wavelength chosen for monitoring to provide the appropriate intensity for all the target compounds.

3.8.3 Chromatographic system for Metronidazole and its surrogates

During analytical method development, the mixtures of methanol and water in different combinations were investigated at different flow rates to determine the effect on elution of Metronidazole and the surrogate reference standards under isocratic conditions. The best separation was obtained by use of water-methanol in the ratio 70:30, as mobile phase at a flow rate of 1.5mL/min, because under these conditions the chromatographic peaks were better defined, better resolved and free from tailing.

An ODS C 18 Phenomenex 250 x 4.6mm column 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, 254nm was the optimum wavelength chosen for monitoring to provide the appropriate intensity for all the target compounds.

3.8.4 Preparation of Mobile Phase

3.8.4.1 Piroxicam and its surrogates

For Piroxicam and its surrogate standards, a mobile phase composition of a phosphate buffer of pH 6.2 and methanol in a ratio of 1:1 was found to give very resolved peaks.

The phosphate buffer was prepared by weighing 13.6100g of potassium dihydrogen orthophosphate into 500mL volumetric flask. It was then dissolved with distilled water in a 500mL volumetric flask. 50mL of the buffer was placed in 200mL volumetric flask and 8.6mL of 0.2M NaOH was added and topped up with water to volume to obtain a pH of 6.2±0.2.

3.8.4.2 Metronidazole and its surrogates

For metronidazole and its surrogate standards, a mobile phase composition of water and methanol in a ratio 70:30 was identified to give good resolution of peaks. To prepare 500mL of this mobile phase 350mL of water was added 150mL of methanol to obtain a 500mL composition of the mobile phase. The solution was then filtered.

3.8.5 Detection of Wavelength of Maximum Absorption

0.001g of each sample was weighed and dissolved with methanol into a 10mL volumetric flask. A quantity of each sample was poured into a cuvette and it was scanned within a wavelength range of 225 to 350nm using a Cecil CE 2041 2000 Series-UV Spectrophotometer.

3.8.6 Stability studies on the drug samples in solution

A known concentration of each of the drug samples used was prepared. The same sample solution was injected six times at an interval of 10 minutes for one hour. Their peak areas were recorded and a graph of peak area against time was plotted to ascertain the stability of the samples in solution within the time of the experiment.

3.9 Analytical Performance Parameters

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

A stock solution of 0.01% w/v of all the samples were prepared and diluted to six different concentrations. Hundred micro-litres (100μL) of the resultant solutions were injected into the column. The peak areas were measured electronically. The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were determined using the following formula;

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

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

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.

3.9.2 Linearity

A stock solution of 0.01% w/v of all the surrogate reference standards were prepared and serially diluted to different concentrations. Hundred micro-litres (100µL) of the resultant solutions were injected into the column. The peak areas were measured electronically and plotted against their respective concentrations.

3.9.3 Reproducibility and Repeatability

The analytes and their respective surrogate reference standards were injected six times in a day on three days. As a result, fresh mobile phases and diluents were prepared, and the analytes as well as the surrogate reference standards were reweighed in accordance with earlier measurements, with the chromatographic conditions maintained throughout.

Within each assay, the analyte as well as the surrogate reference standard injections were repeated to ascertain the precision between the different sets of assay carried out within the day for a particular sample.

3.9.4 Accuracy and Precision

The percentage contents obtained from the standard method and the new method were subjected to various tests to investigate their accuracy and precision. The various tests are Relative Standard Deviation, Standard Error of Mean, F-test and T-test.

3.10 Determination of K using the surrogate reference standards

A stock solution of 0.01%w/v of a surrogate reference standard as well as the analyte was prepared. Various concentrations of the surrogate reference standard as well as the analyte were prepared. Five milliliters of a surrogate reference standard as well as five milliliters of the analyte were mixed and 100µL of the resulting solution injected. Chromatograms were recorded and peak areas measured electronically. The constant K for each surrogate reference standard against the analyte was calculated using the relation for K stated in the hypothesis of this thesis.

3.11 Analysis of Commercial Samples using the Surrogate Reference Standards

3.11.1 Piroxicam capsules

Twenty tablets of each of the four different brands of Piroxicam capsules were powdered. 0.1315g of the powder containing 10mg pure Piroxicam was dissolved in 10mL of the diluents ie the mobile phase and sonicated for 15minutes. 1mL of the resulting solution was subsequently dissolved in 10mL of the same diluent. A 2ml portion of the resulting solution was taken and dissolved in 10ml with the same diluents. A portion of the resulting solution was filtered using a micro filter. A concentration 0.005%w/v of the surrogate reference standard was also prepared and filtered using a micro filter. 5mL each of the analyte and the surrogate reference standard were mixed and 100µL of the resulting solution was injected and the corresponding peak area was recorded from the chromatograph.

3.11.2 Metronidazole tablets

Twenty tablets of each of the four different brands of Metronidazole tablets were powdered. A quantity of the powder containing 10mg pure Metronidazole were each dissolved in 10mL of the diluents ie the mobile phase. 1mL of the resulting solution was subsequently dissolved in 10mL

of the same diluent. A portion of the resulting solution was filtered using a micro filter. A known concentration of the surrogate reference standard was also prepared and filtered using a micro filter. 5mL each of the analyte and the surrogate reference standard were mixed and 100µL of the resulting solution was injected and the corresponding peak area was recorded from the chromatograph.

CHAPTER FOUR

4.0 RESULTS AND CALCULATIONS

4.1 Identification of pure samples

4.1.1 Qualitative tests

4.1.1.1 Results of Colour and UV tests

Table 4.1 Results of tests

Sample	Result	Inference
Metronidazole	1. The solution showed an absorption maximum at 277 nm and a minimum at 240 nm. The specific absorbance at the maximum was 390.	Positive (BP)
Ascorbic acid	1. The absorbance of the solution was measured at 243nm immediately after dissolution. The specific absorbance at the maximum is 578. 2. The pH of the solution is 2.4. 3. A grey precipitate was formed	Positive (BP) Positive (BP) Positive (BP)

4.1.1.2 Melting point determination

Table 4.2 Results of Melting point determination

Sample	Literature Value (BP) (^{O}C)	Experimental Value (^{O}C)
Piroxicam	240 - 245	241 - 244
Metronidazole	159 - 163	161 - 163
Metformin	222 - 226	222 - 224
Ascorbic acid	190 - 192	190- 191
Para aminophenol	186	185 - 188

4.1.2 Assay of pure samples

4.1.2.1 Piroxicam pure sample

Table 4.3 Titration results for Piroxicam

Sample	A	B	C	Blank
Weight taken	0.2503	0.2500	0.2501	
Endpoint	7.70	7.60	7.70	0.10

Titre value = 7.7mL – Blank (0.1mL) = 7.6mL

Factor of perchloric Acid = 0.9931

Actual titre = 7.6 x 0.9931

= 7.5476mL

1 mL of 0.1 M perchloric acid is equivalent to 0.03314g of $C_{15}H_{13}N_3O_4S$.

Actual amount of Piroxicam = 7.5476 x 0.03314g

= 0.2501g

Thus the percentage purity (%) = (0.2501/0.25) x 100

= 100.05%

Similar calculations were done to obtain percentage contents 98.73 and 100.05 for samples B and C respectively.

Therefore average percentage content is the calculated as follows;

Average percentage content = (100.05 + 98.73 + 100.05)/ 3

= 99.61%

Table 4.4 Results of Average Percentage Purities of samples

Sample	**Av. Percentage Purity**
Piroxicam	99.61
Metronidazole	99.74
Metformin	100.32
Ascorbic acid	100.00

4.1.3 Thin Layer Chromatography (TLC)

4.1.3.1 TLC for Piroxicam capsules

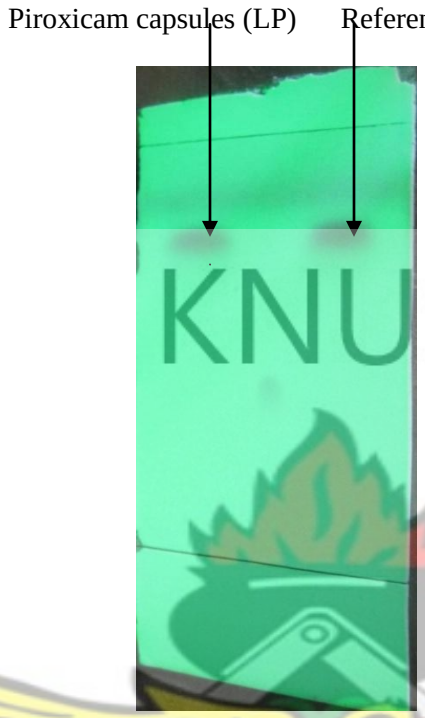

Figure 4.1 Piroxicam capsules from Letap Pharmaceuticals

Rf Value = Distance travelled by substance from the origin
Distance travelled by the solvent front from the origin

Piroxicam capsules from Letap Pharmaceuticals Limited, Ghana

Rf = 4/4.6

= 0.870

Reference

Rf = 4/4.6

= 0.870

4.1.3.2 TLC for Metronidazole tablets

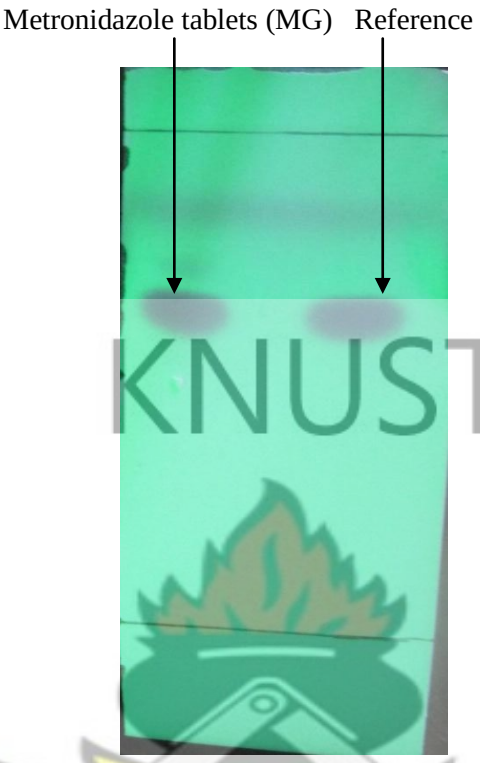

Figure 4.2 Metronidazole tablets from M&G Pharmaceuticals

Rf Value = Distance travelled by substance from the origin
Distance travelled by the solvent front from the origin

Metronidazole tablets from M & G Pharmaceuticals Limited, Ghana

Rf = 3.7/4.6

= 0.804

Reference

Rf = 3.7/4.6
= 0.804

Table 4.5 Results of Rf values of Piroxicam pure sample and capsules

Sample	Name of Manufacturer	Rf Value for pure sample (reference)	Rf value for capsules
Piroxicam	Ernest chemist Limited	0.880	0.880
Piroxicam	Kinapharma Limited	0.875	0.875
Piroxicam	Letap Pharmaceuticals	0.870	0.870
Piroxicam	Luex Pharmaceuticals Limited	0.875	0.875

Table 4.6 Results of Rf values of Metronidazole pure sample and tablets

Sample	Name of Manufacturer	Rf Value for pure sample (reference)	Rf value for Tablets
Metronidazole	Ernest chemist Limited	0.792	0.792
Metronidazole	M&G Pharmaceuticals Ltd	0.804	0.804
Metronidazole	Letap Pharmaceuticals	0.800	0.800
Metronidazole	Maxheal Laboratories Limited	0.795	0.795

4.2 Calculation of Factors

4.2.1 Calculation of Factor for Iodine using Sodium Thiosulphate

Amount of $Na_2S_2O_3$ weighed = 2.4800g

Factor of $Na_2S_2O_3$ = Actual weight / Nominal weight

= 2.4800/2.4800

= 1.0000

Volume of I_2= 25.50mL

Volume of $Na_2S_2O_3$= 25mL

Factor (I_2) = [Factor ($Na_2S_2O_3$) × Volume ($Na_2S_2O_3$)] / Volume (I_2)

= (1.0000 x 25)/ 25.5 = 0.9804

4.2.2 Calculation of Factor for 0.1M Perchloric acid using Potassium Hydrogen Phthalate

Amount of $C_8O_4H_5K$ weighed = 0.5005g

Factor of $C_8O_4H_5K$ = Actual weight / Nominal weight

= 0.5005/0.5

= 1.001

Volume of $HClO_4$= 25.20mL

Volume of $C_8O_4H_5K$ = 25mL

Factor ($HClO_4$) = [Factor ($C_8O_4H_5K$) × Volume ($C_8O_4H_5K$)] / Volume ($HClO_4$)

= (1.001 x 25)/ 25.20

= 0.9931

4.3 Uniformity of weight

Refer to table U1 to U8 in the appendix

4. 4 Assay of Piroxicam capsules and Metronidazole tablets using standard methods

4. 4.1 Determination of percentage content of Piroxicam in Piroxicam capsules (20mg) using the Standard method (BP 2007)

Product: Piroxicam Capsules manufactured by Ernest Chemist, Ghana

0.02g of pure Piroxicam is equivalent to 0.2630g of Piroxicam capsules

Therefore 0.01g of pure Piroxicam will be equivalent to 0.1315g of piroxicam capsules

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

Where, Aa is the peak area of the Piroxicam capsules (analyte)

As is the peak area of the piroxicam pure sample (standard)

Cs is the concentration of the piroxicam pure sample (standard)

Ca is the concedntration of the piroxicam capsules (analyte)

Therefore,

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

Aa = 15.66

As = 16.17

Cs = 0.005%w/v

$$Cs = \frac{15.66 \times 0.005}{16.17}$$

= 0.00484%w/v

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

$$= \frac{0.00484 \times 100}{0.005}$$

= 96.85%

Table 4.7 Results obtained from the analysis of Piroxicam capsules

Manufacturing company	**Batch number**	**Percentage Content**					
		1	**2**	**3**	**4**	**5**	**Mean Percentage content**
Ernest Chemist	0508J	95.95	96.40	94.85	96.85	96.78	96.17±0.37
Kinapharma	10012	99.52	100.95	99.84	98.89	98.74	99.59±0.40
Letap	0210131	100.12	103.01	101.12	100.98	101.45	101.34±0.47
Luex	100428	98.14	97.24	99.14	97.87	100.11	98.50±0.51

Table 4.8 Results obtained from the analysis of Metronidazole tablets

Manufacturing company	Batch number	Percentage Content					
		1	2	3	4	5	Mean Percentage content
Ernest Chemist	0701L	97.01	99.02	98.32	97.70	98.39	98.08±0.34
Letap	0740881	103.33	103.70	104.98	104.93	103.81	104.15±0.34
M & G	MZ119	97.29	98.58	95.96	97.97	96.58	97.44±0.47
Maxheal Lab. Ltd	XT015	104.38	105.29	105.85	104.16	104.41	104.82±0.32

4.5 HPLC method developlment

4.5.1 UV Spectrum of samples

Table 4.9 Wavelength of Maximum Absorption of Samples

Sample	Wavelength range (nm) of Absorbance	Wavelength of Maximum absorbance
Piroxicam	220 - 350	239.5, 325.5
Metformin	220 - 350	236
Metronidazole	220 – 350	310
Ascorbic Acid	220 – 350	245

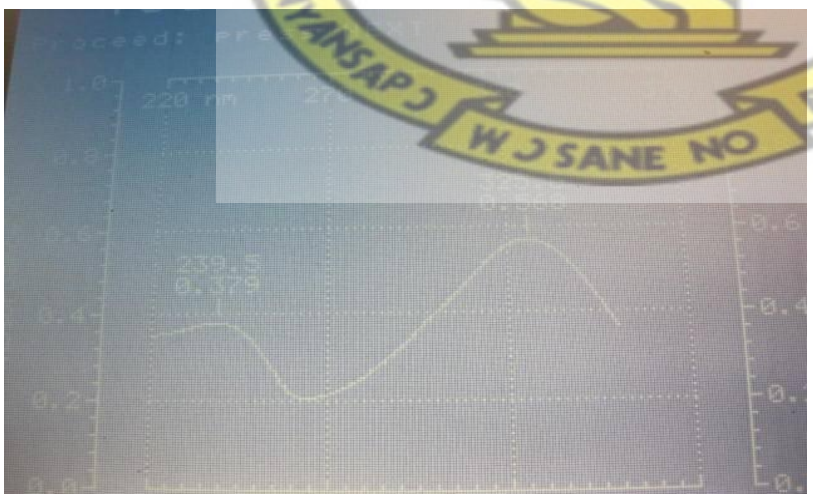

Figure 4.3 A UV spectrum of Piroxicam pure sample

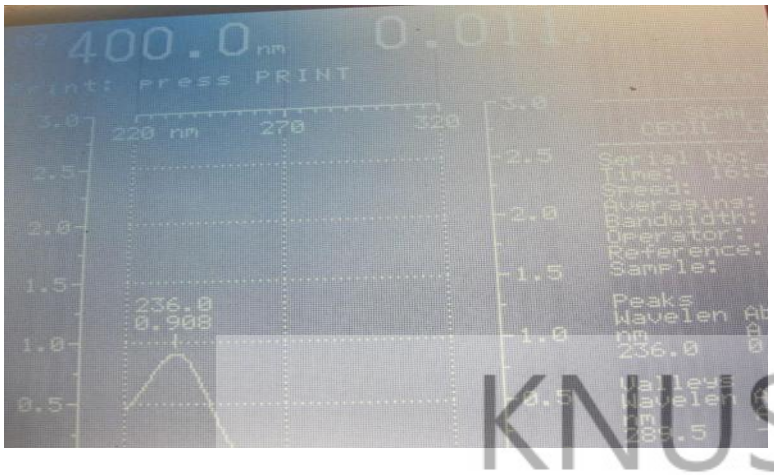

Figure 4.4 A UV spectrum of Metformin pure sample

Figure 4.5 A UV spectrum of Metronidazole pure sample

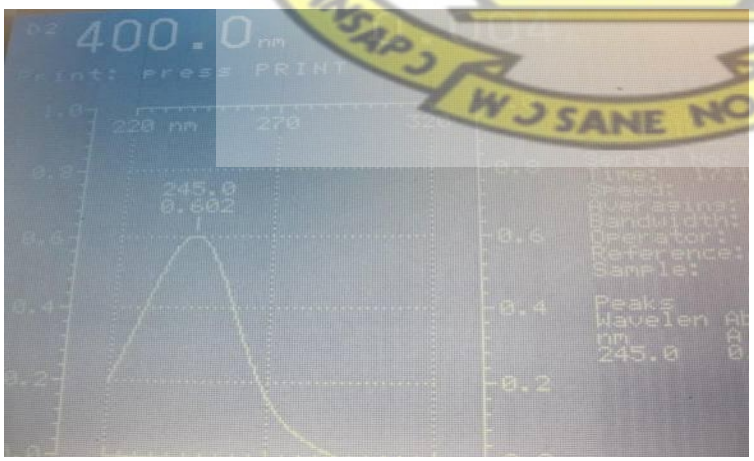

Figure 4.6 A UV spectrum of Ascorbic acid pure sample

4.5.2 Chromatographic conditions for the Analysis of Piroxicam Capsules and Metronidazole Tablets

The HPLC conditions used for the analysis of Piroxicam and Metronidazole are as indicated in the table below;

Table 4.10 Chromatographic Conditions for Piroxicam and Metronidazole and their surrogates

Parameter	**Piroxicam**	**Metronidazole**
Mobile phase	1:1 methanol : phosphate buffer pH 6.2	water: methanol (70: 30)
Stationary Phase	ODS C 18 Phenomenex 250 x 4.6mm column	ODS C 18 Phenomenex 250 x 4.6mm column
Detector	UV-Visible detector	UV-Visible detector
Wavelength	254nm	254nm
Flow rate	1mL/min	1.5mL/mL
Sensitivity	0.050	0.050
Injector volume	100µL	100µL

Table 4.11 Mean retention time for Piroxicam and its surrogates

Sample	**Mean Retention time**
Piroxicam	6.83±0.065
Metronidazole	4.07±0.058
Metformin	3.42±0.049
Ascorbic acid	2.56±0.055

Table 4.12 Mean retention time for Metronidazole and its surrogates

Sample	Mean Retention time
Metronidazole	4.24±0.014
Ascorbic acid	1.80±0.012
Para aminophenol	2.82±0.015

4.5.3 Chromatograms

4.5.3.1 Piroxicam and its surrogates

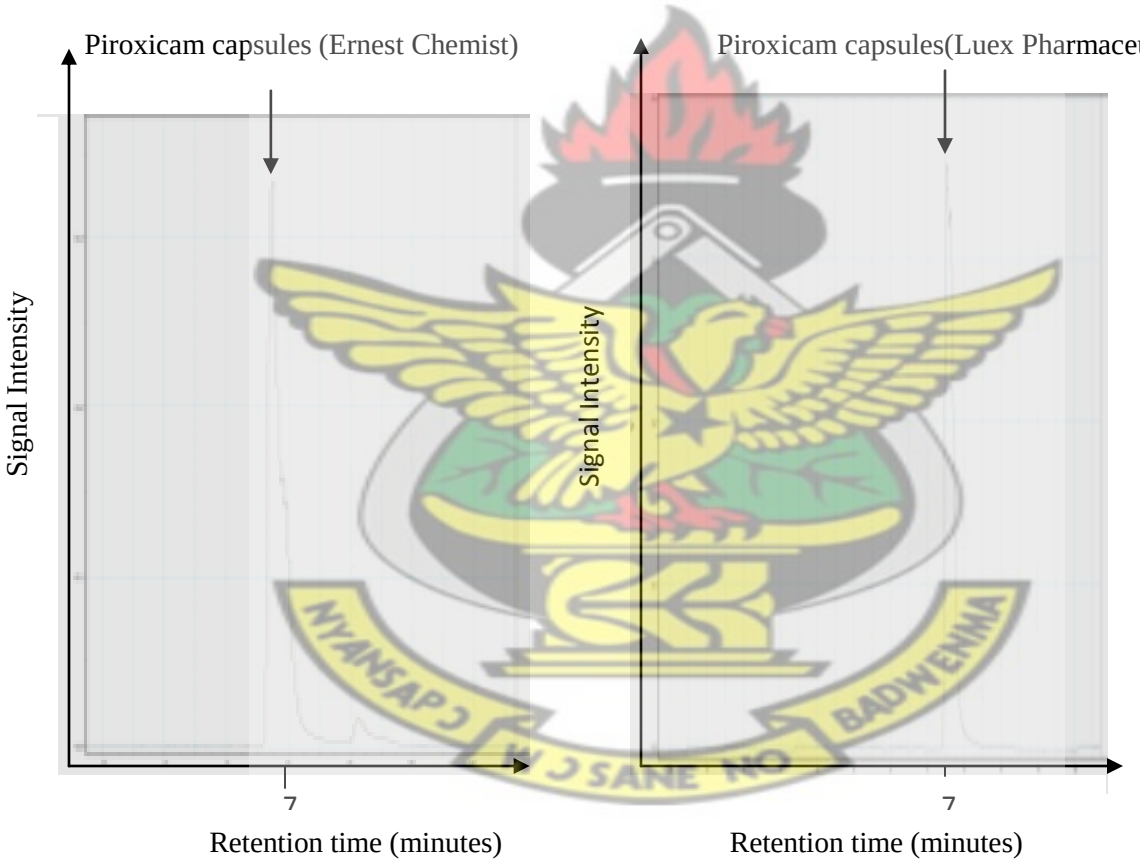

Figure 4.7 Chromatogram of Piroxicam capsules from Ernest Chemist

Figure 4.8 Chromatogram of Piroxicam capsules from Luex Pharmaceuticals

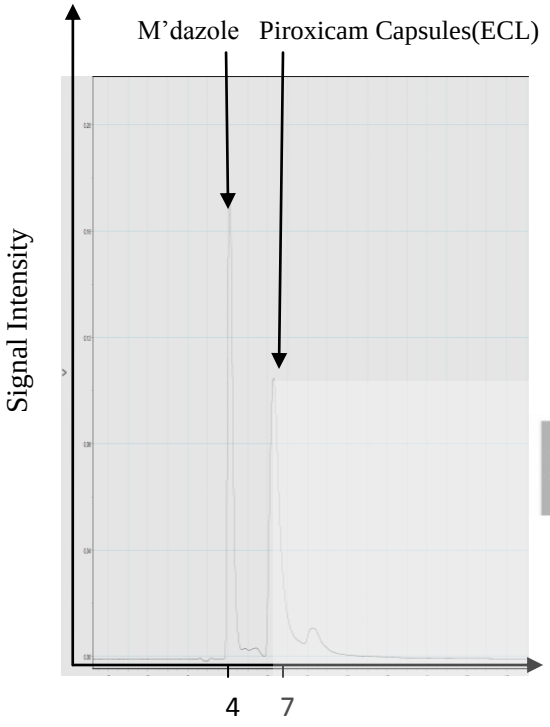

Figure 4.9 Chromatogram of Piroxicam capsules from Ernest Chemist Limited and Metronidazole pure sample (Surrogate)

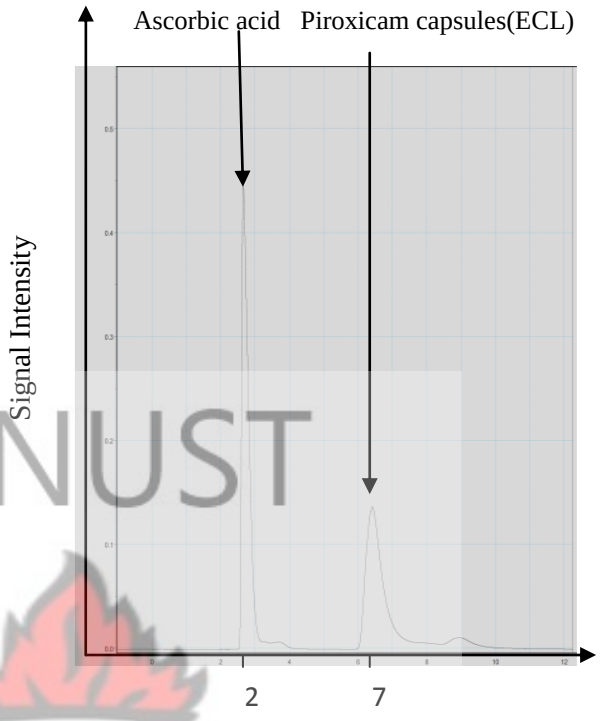

Figure 4.10 Chromatogram of Piroxicam capsules from Ernest Chemist and Ascorbic acid pure sample (Surrogate)

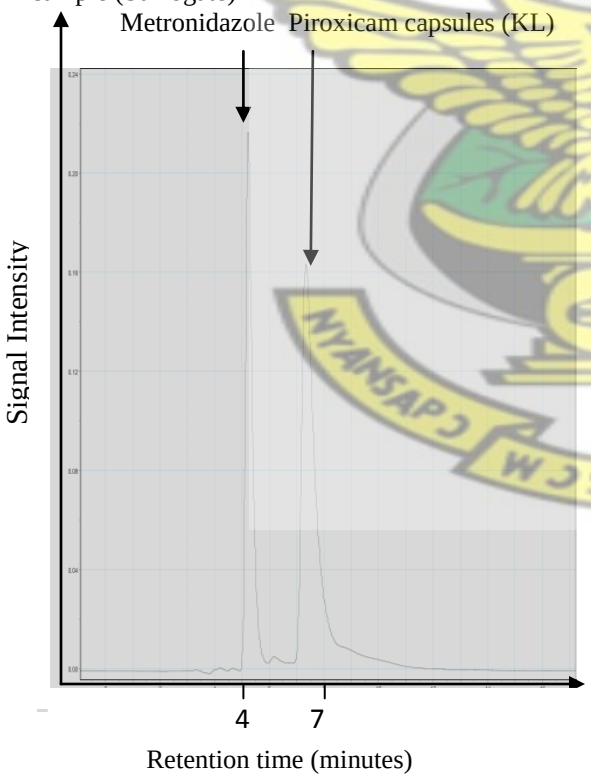

Figure 4.11 Chromatogram of Piroxicam capsules from Kinapharma Limited and Metronidazole pure sample (Surrogate)

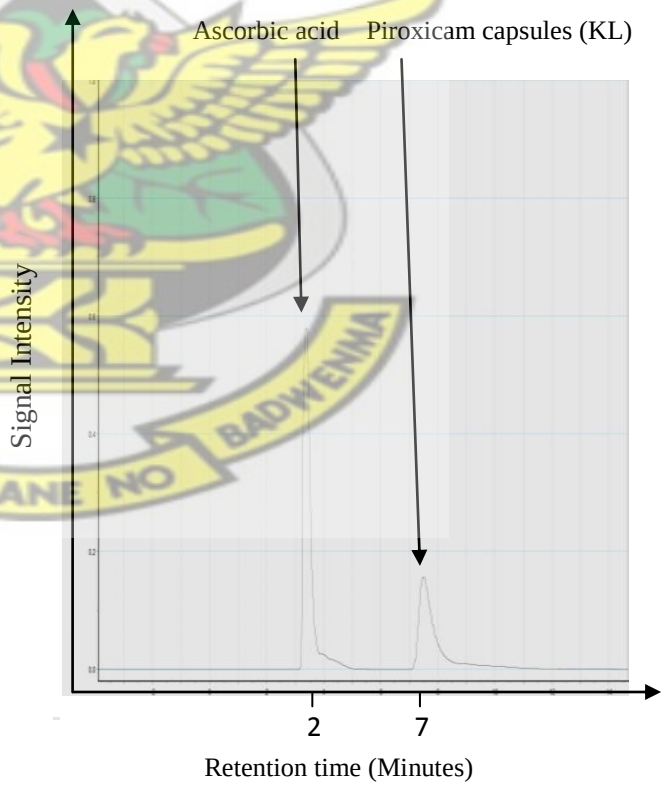

Figure 4.12 Chromatogram of Piroxicam capsules from Kinapharma Limited and Ascorbic acid pure (Surrogate)

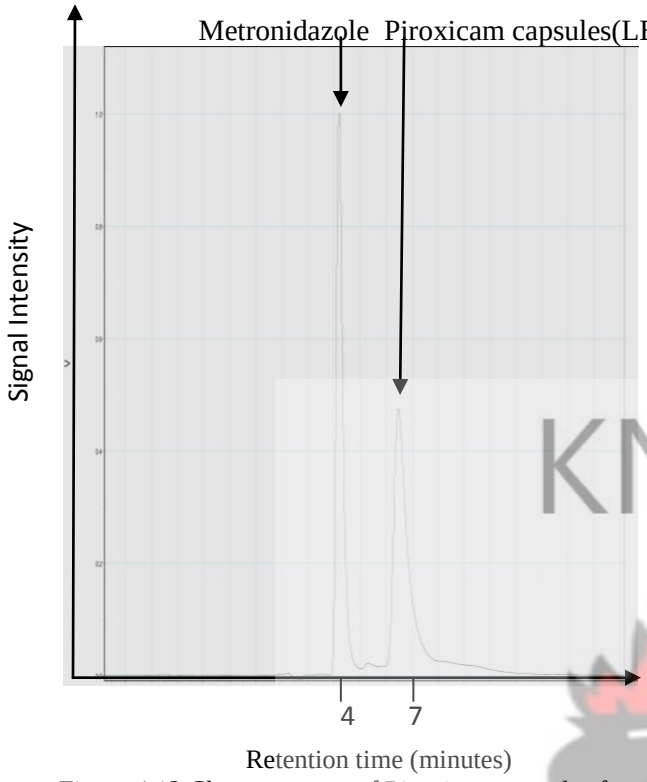

Figure 4.13 Chromatogram of Piroxicam capsules from Letap Pharmaceuticals and Metronidazole pure sample (Surrogate)

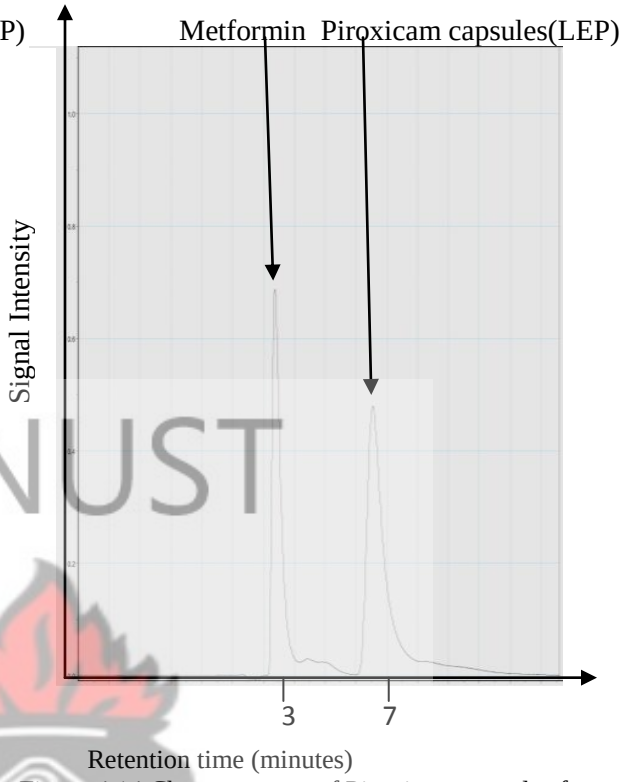

Figure 4.14 Chromatogram of Piroxicam capsules from Letap Pharmaceuticals and Metformin pure sample (Surrogate)

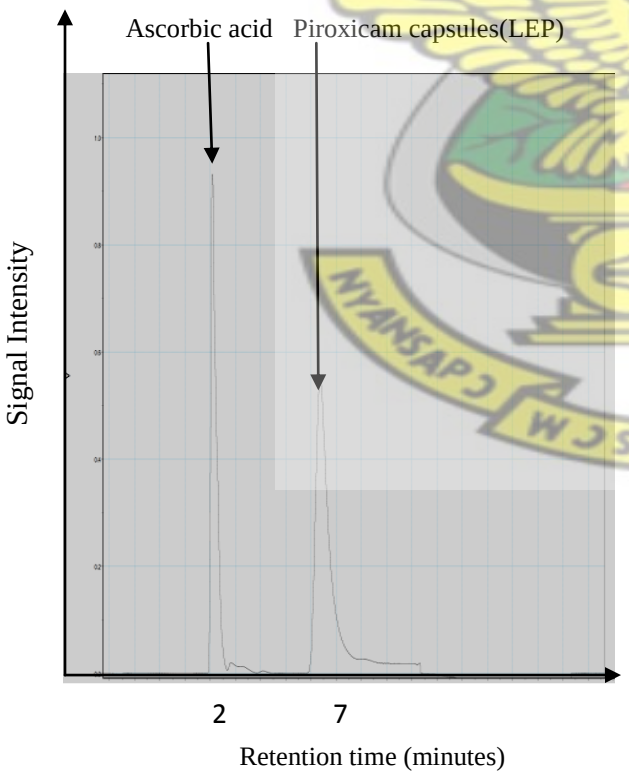

Figure 4.15 Chromatogram of Piroxicam capsules from Letap Pharmaceuticals and Ascorbic acid pure (Surrogate)

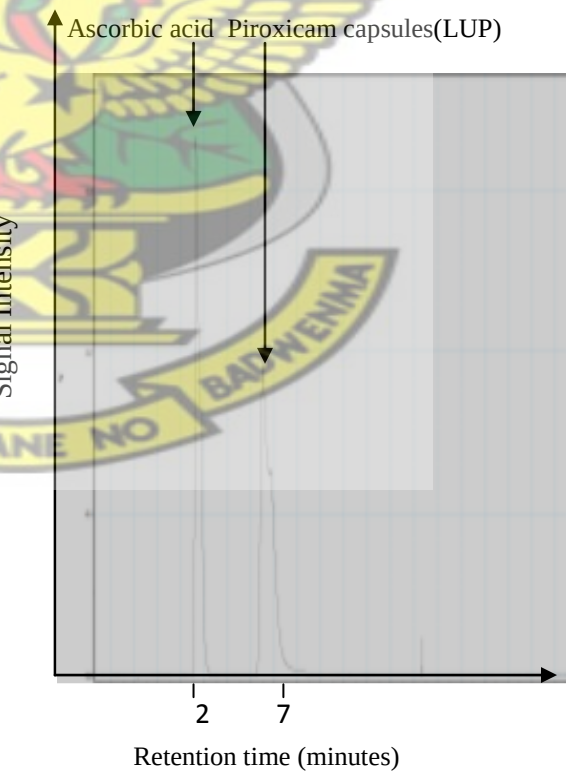

Figure 4.16 Chromatogram of Piroxicam capsules from Luex Pharmaceuticals and Ascorbic acid (Surrogate)

4.5.3.2 Metronidazole and its surrogates

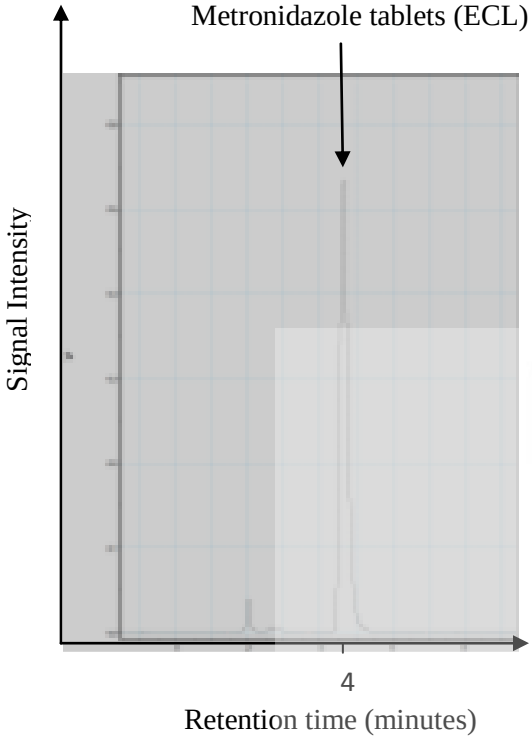

Fig 4.17 Chromatogram of Metronidazole tablets Ernest Chemist Limited

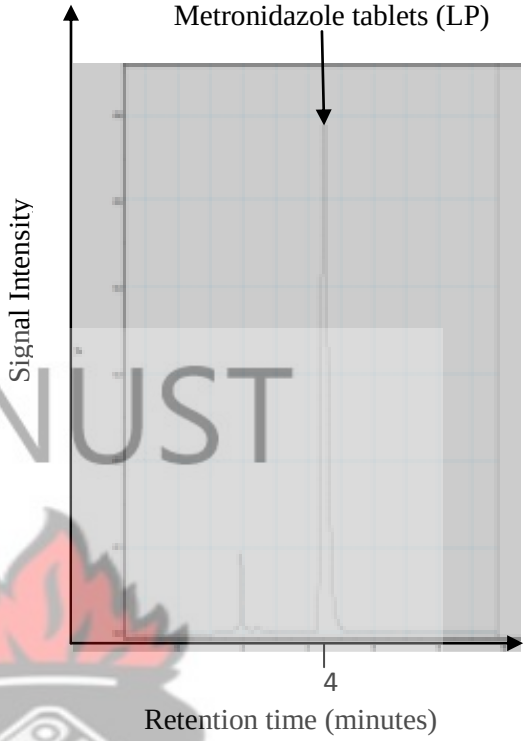

Fig 4.18 Chromatogram of Metronidazole tablets from Letap Pharmaceuticals

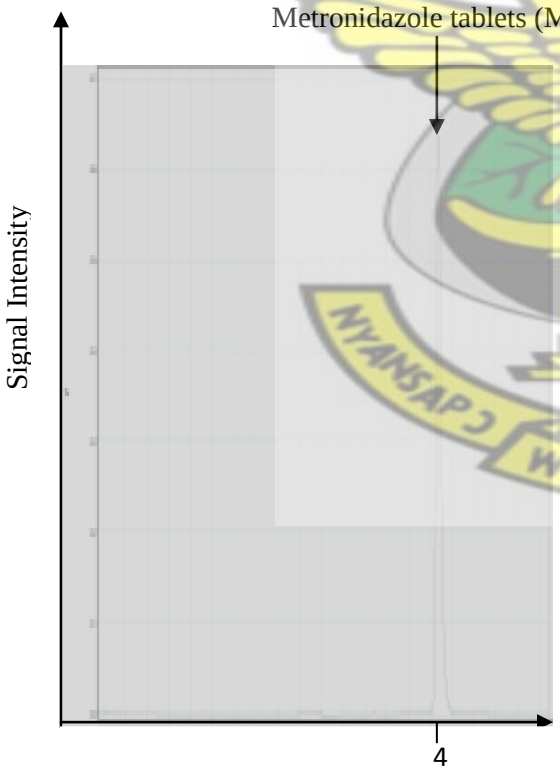

Fig 4.19 Chromatogram of Metronidazole tablets from M&G Pharmaceuticals.

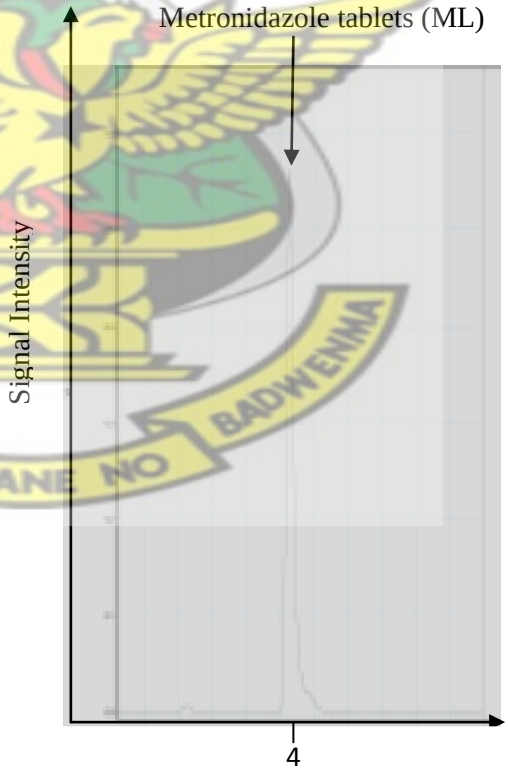

Fig 4.20 Chromatogram of Metronidazole tablets from Maxheal Laboratories Limited

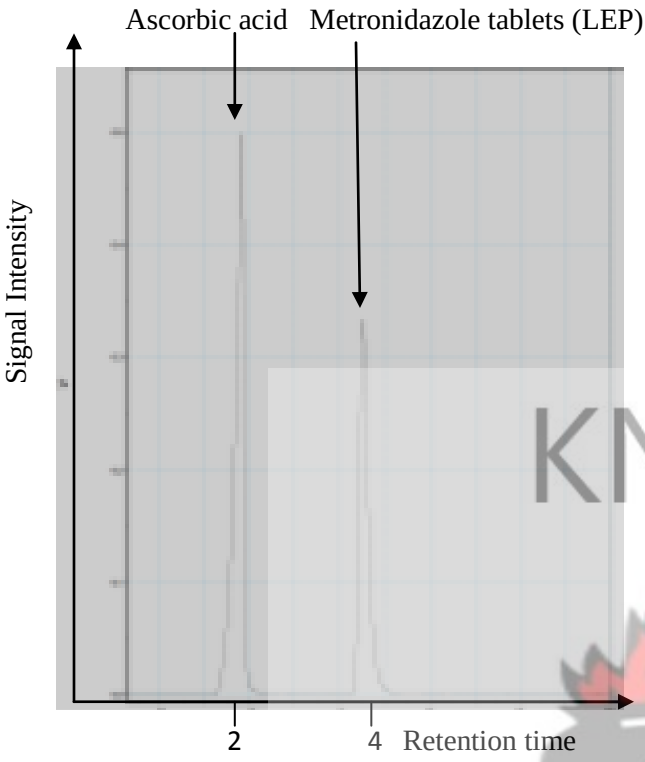

Figure 4.21 Chromatogram of Metronidazole tablets From Letap Pharmaceuticals and Ascorbic acid pure (Surrogate)

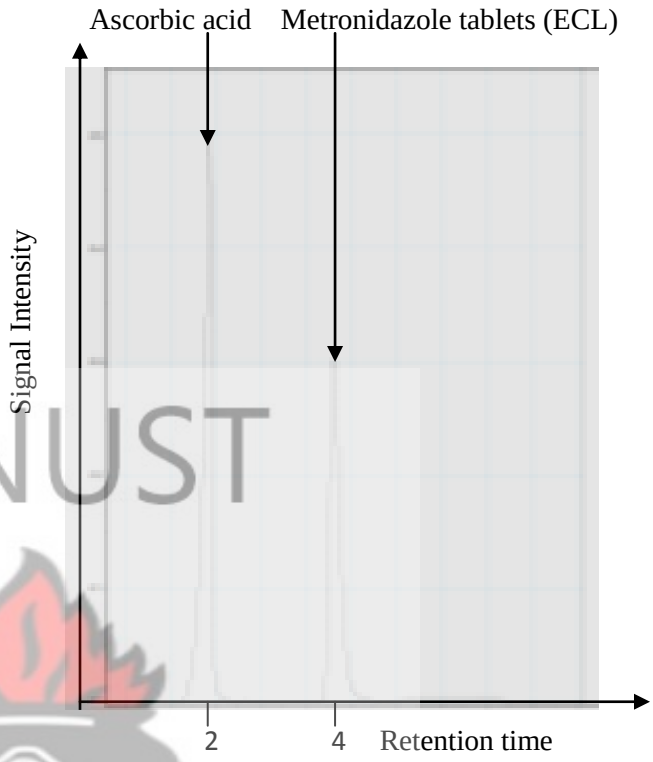

Figure 4.22 Chromatogram of Metronidazole tablets from Ernest Chemist and Ascorbic acid pure (surrogate)

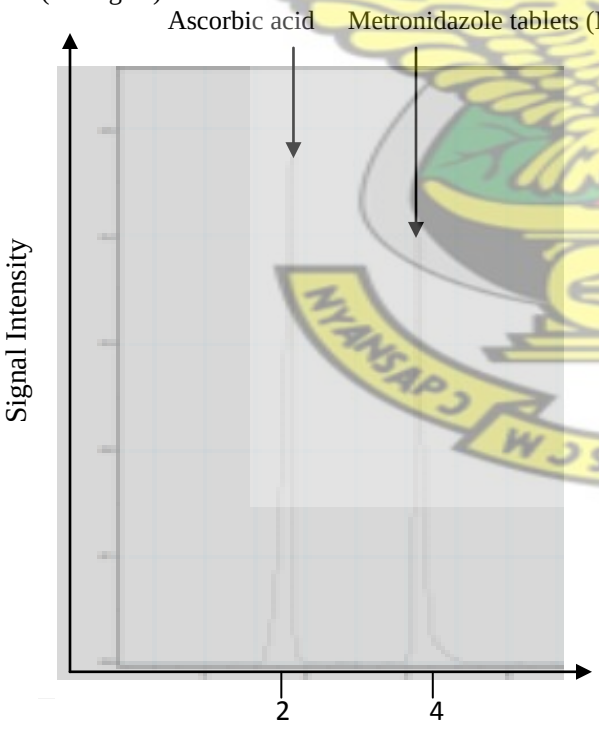

Figure 4.23 Chromatogram of Metronidazole tablets from M & G Pharmaceuticals and Ascorbic acid pure (Surrogate)

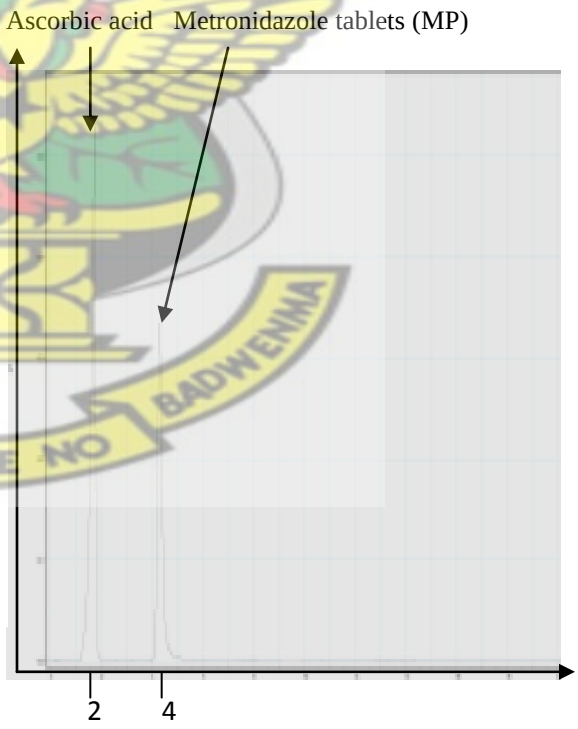

Figure 4.24 Chromatogram of Metronidazole tablets from Maxheal Laboratories and Ascorbic acid pure (surrogate)

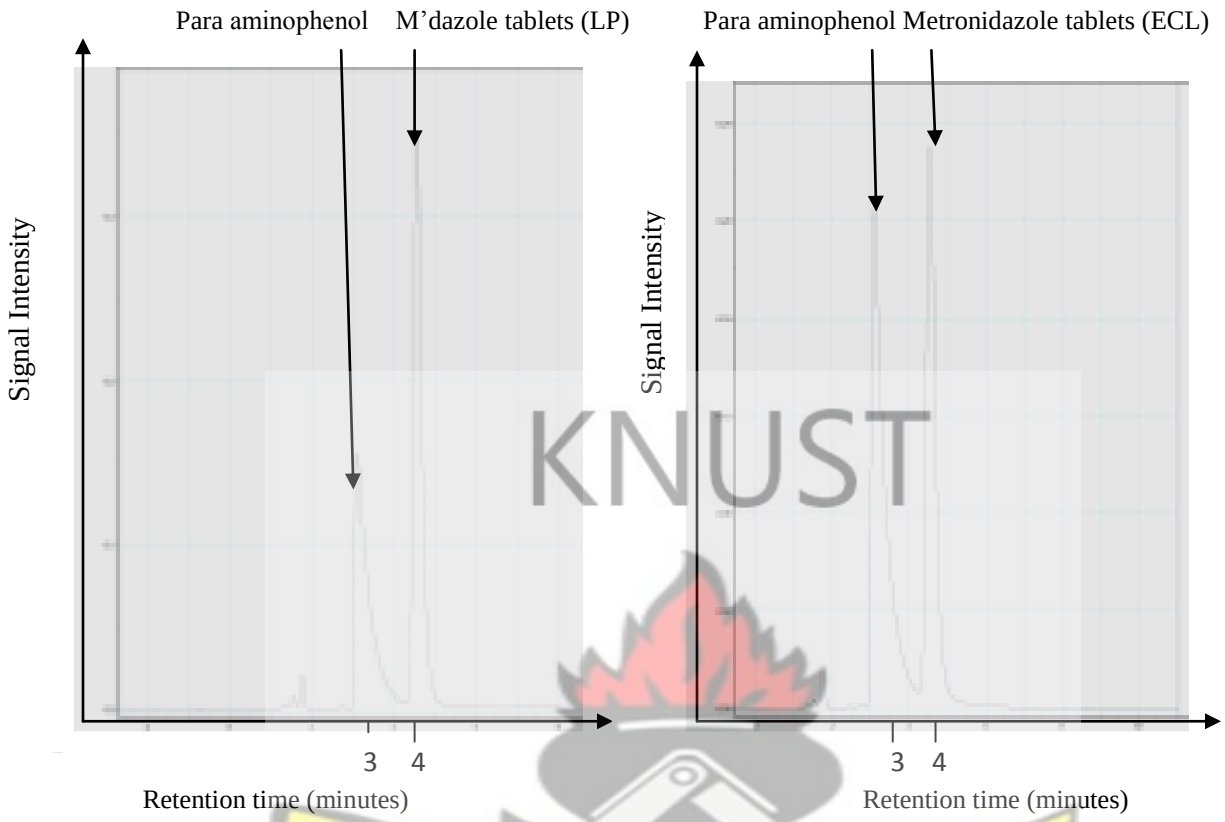

Figure 4.25 Chromatogram of Metronidazole tablets from Letap Pharmaceuticals and Para aminophenol pure (Surrogate)

Figure 4.26 Chromatogram of Metronidazole tablets from Ernest Chemist and Para aminophenol pure sample (Surrogate)

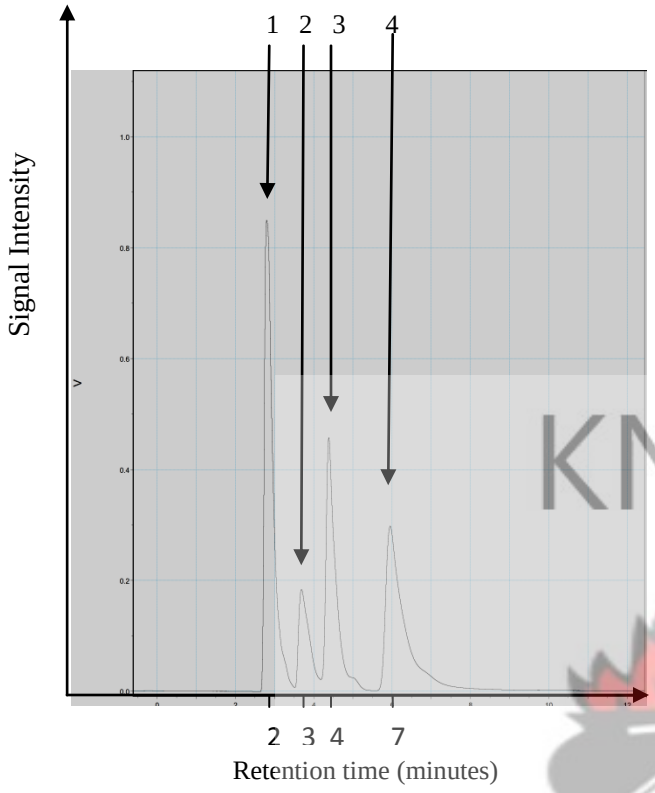

Fig 4.27 Chromatogram of Piroxicam, Metronidazole, Metformin and Ascorbic acid

1 ….. Ascorbic acid 2…… Metformin 3……Metronidazole 4……Piroxicam

4.5.4 Calibration curves

4.5.4.1 Calibration curves for Piroxicam and its surrogates

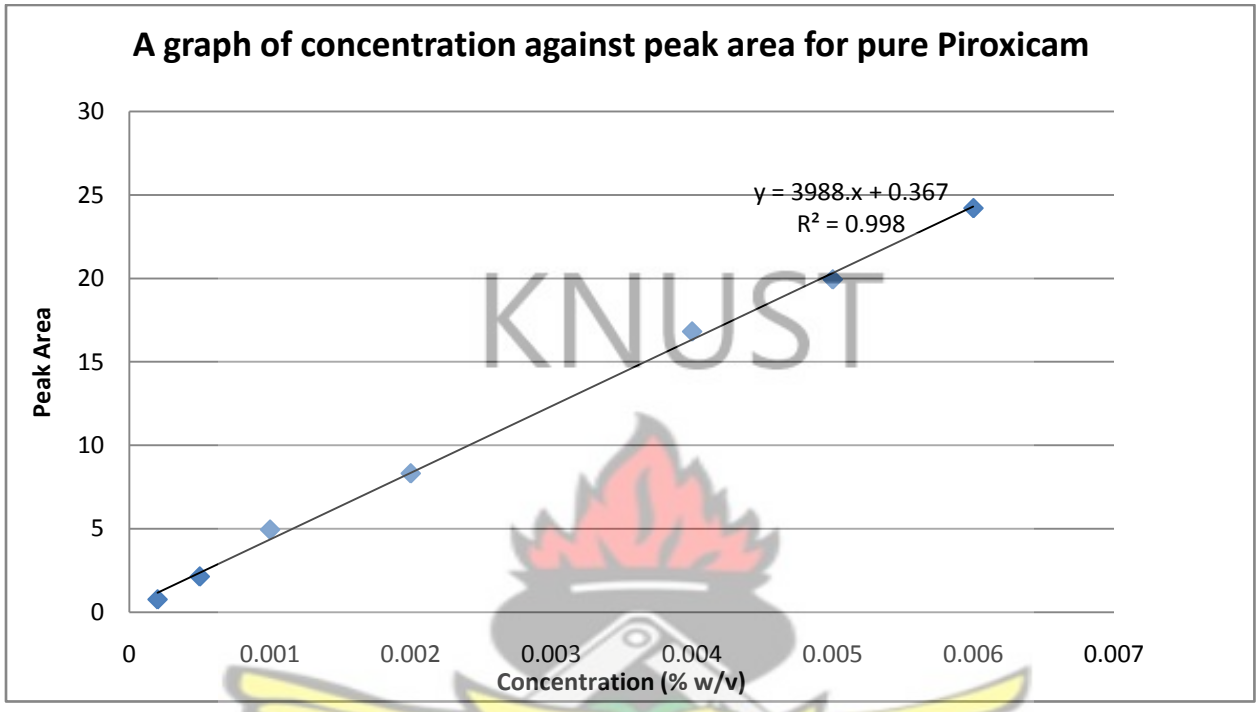

Fig 4.28 Calibration Curve of Pure Piroxicam

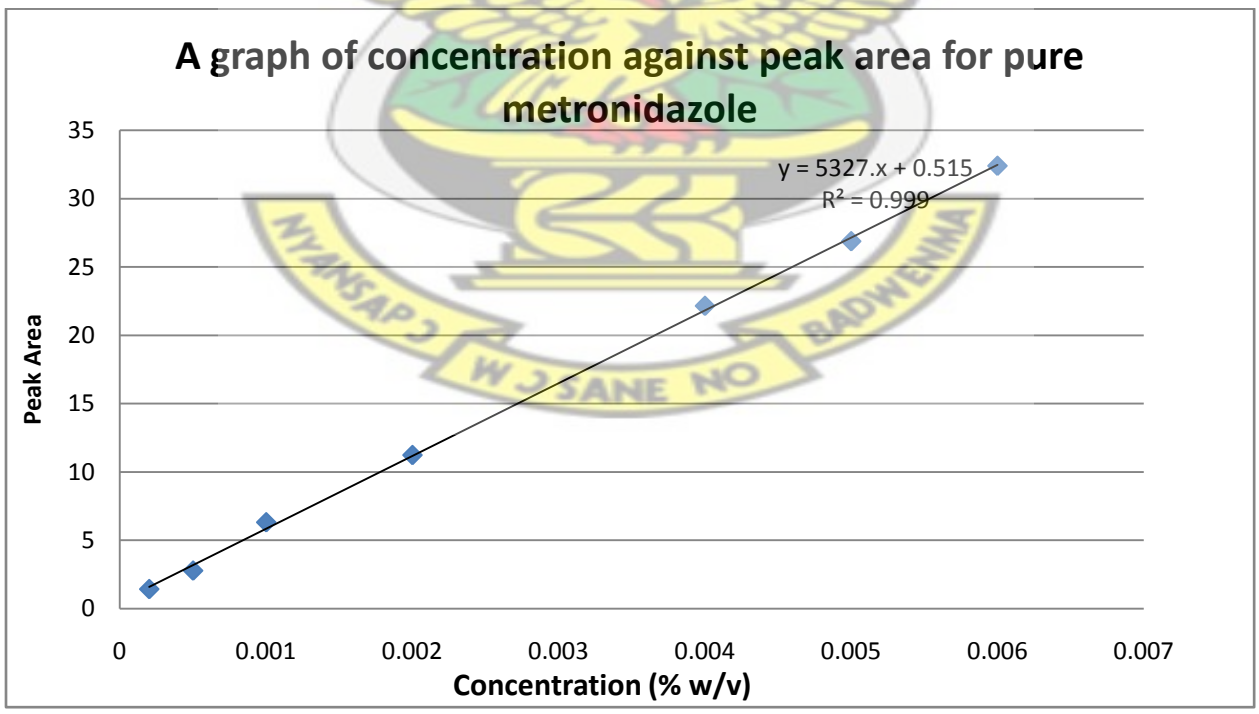

Fig 4.29 Calibration Curve of Pure Metronidazole

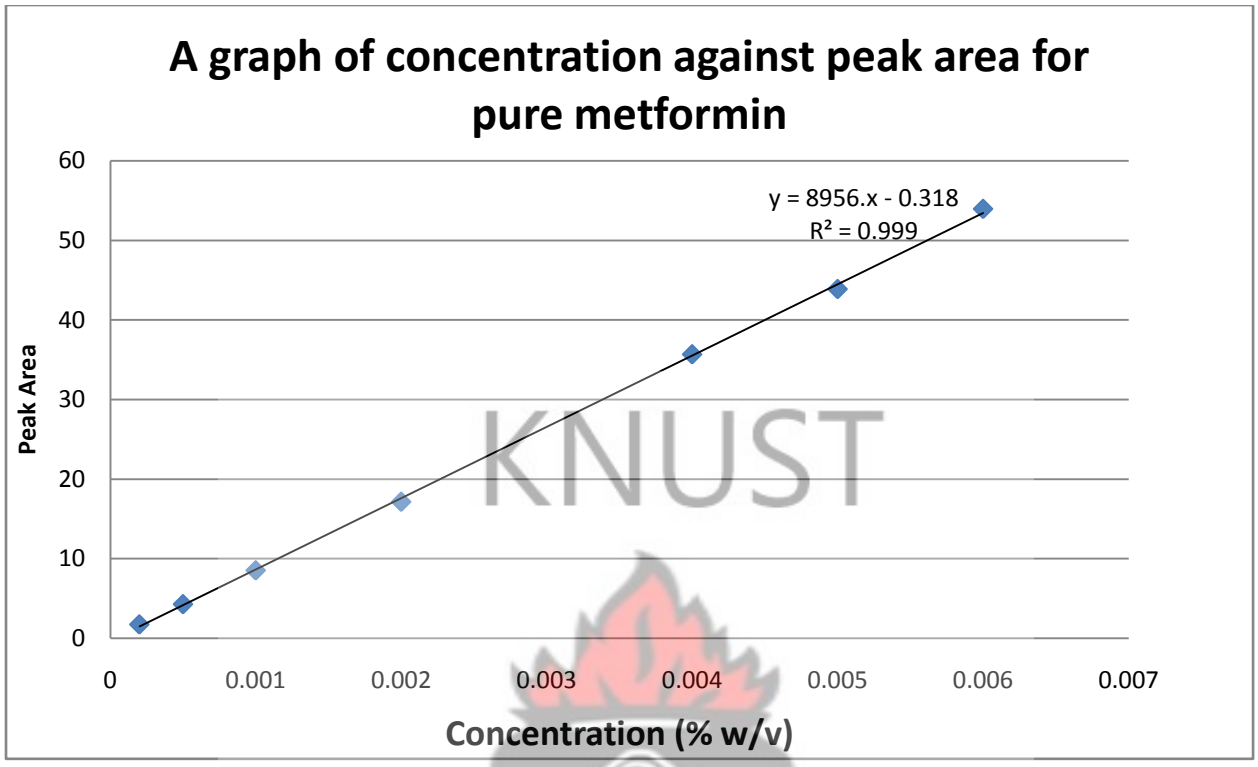

Fig 4.30 Calibration Curve of Pure Metformin

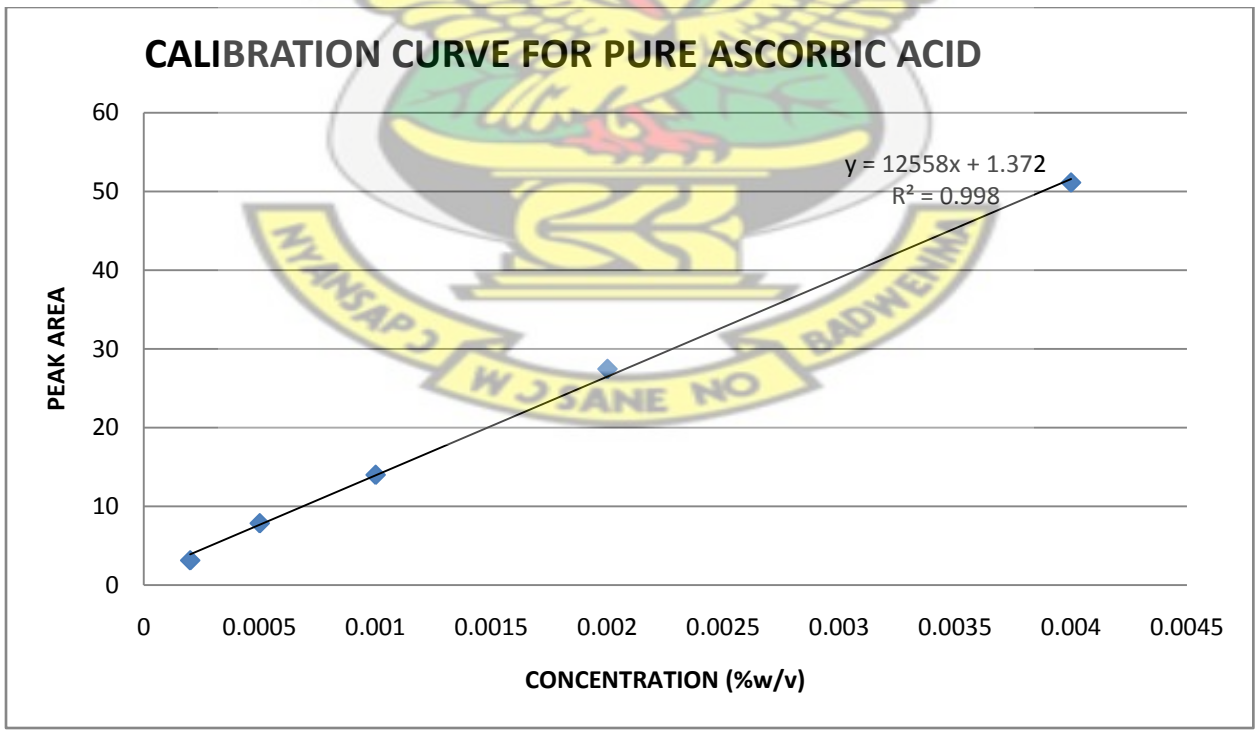

Fig 4.31 Calibration Curve of Pure Ascorbic acid

4.5.4.2 Calibration curves for Metronidazole and its surrogates

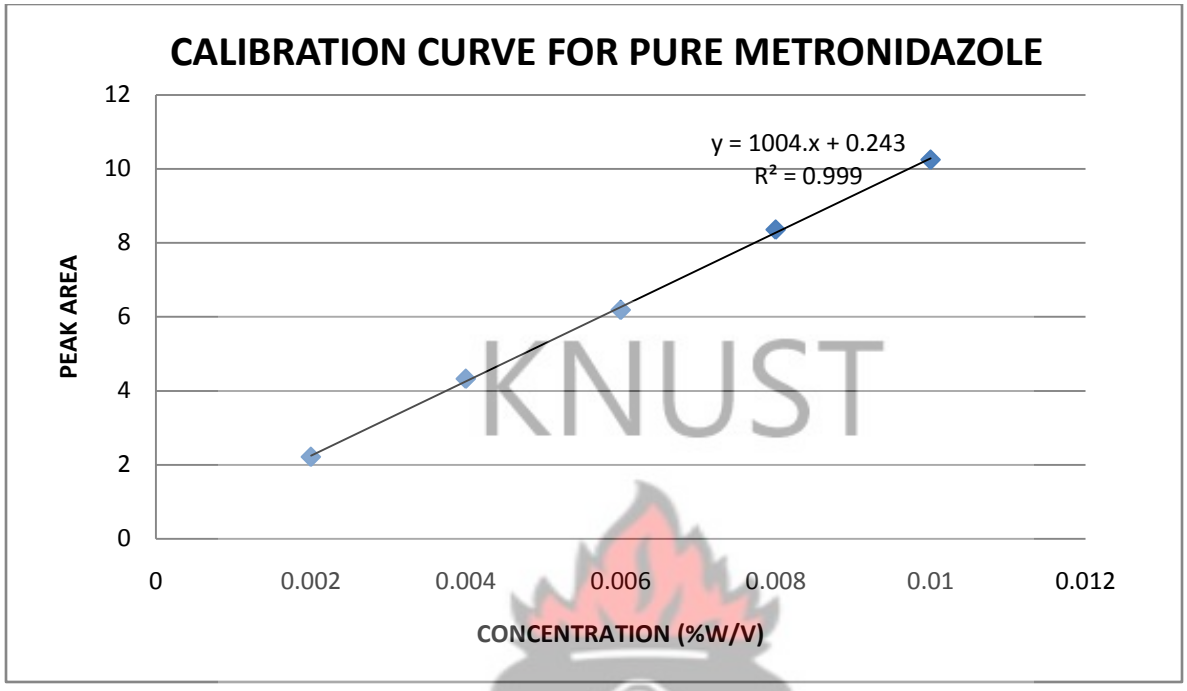

Fig 4.32 Calibration Curve of Pure Metronidazole

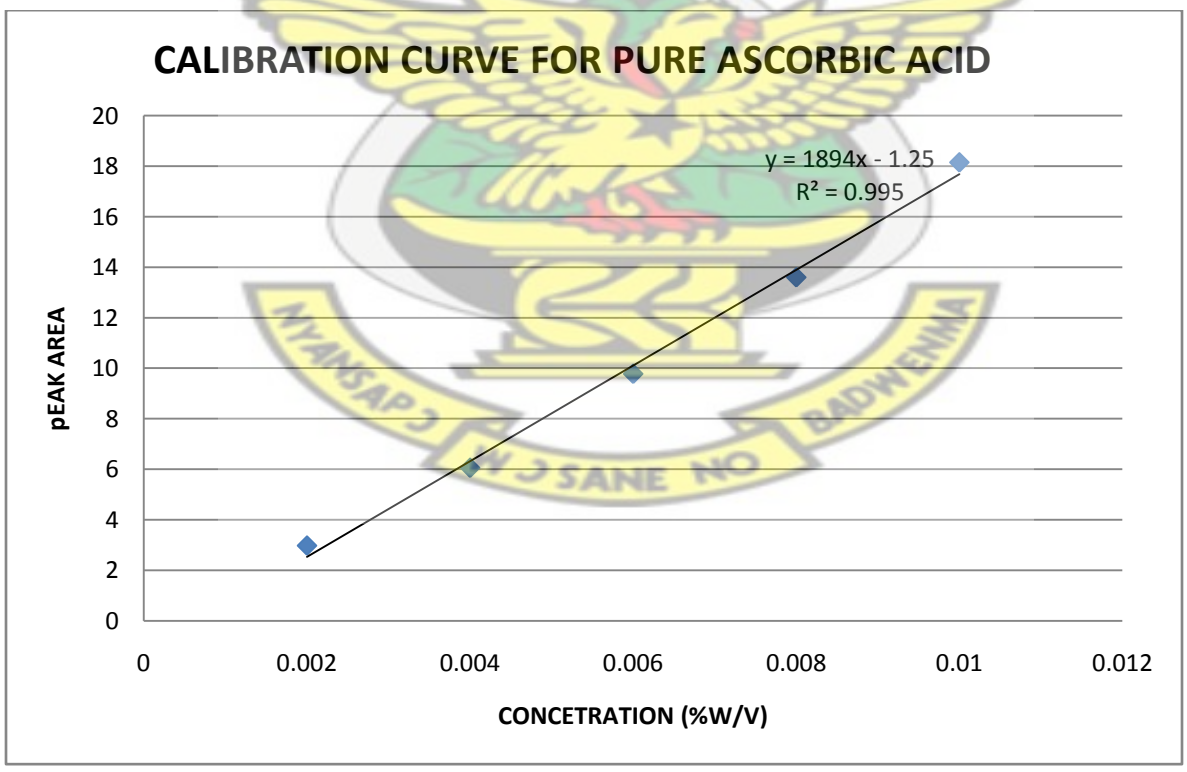

Fig 4.33 Calibration Curve of pure Ascorbic acid

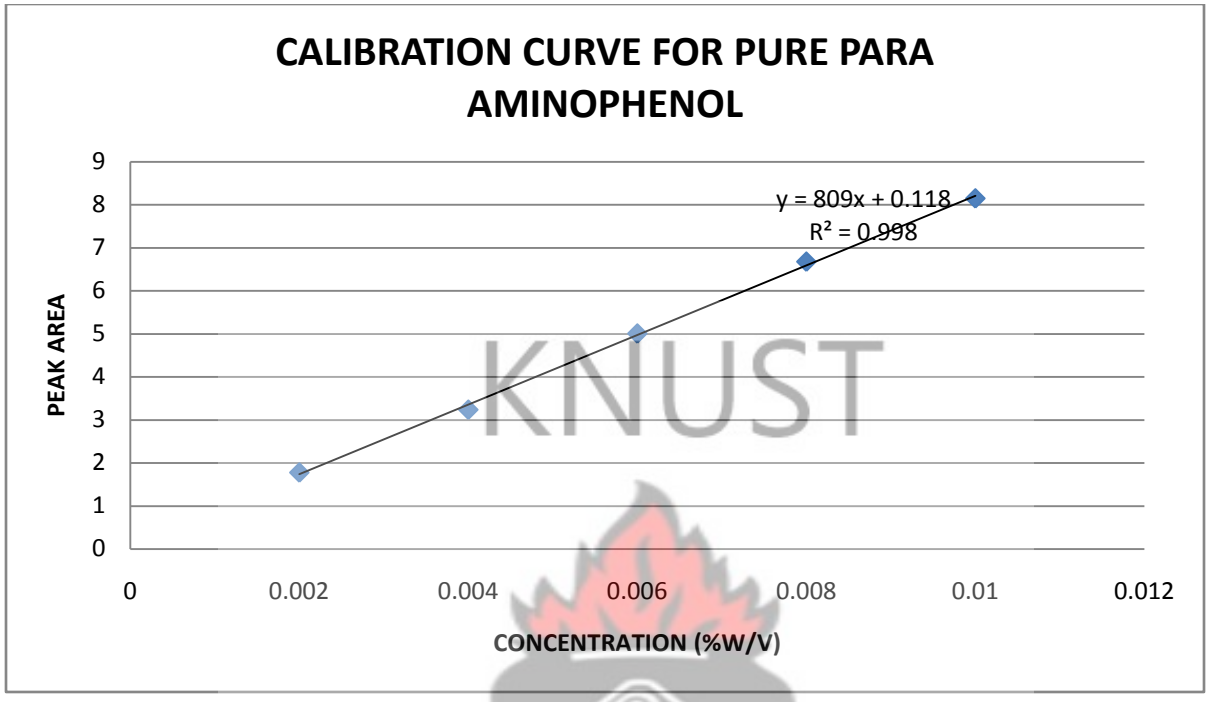

Fig 4.34 Calibration Curve of Pure Para aminophenol

4.6 Analytical Performance Parameters

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

LOD = 3.3σ / S and LOQ = 10σ / S

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

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.

4.6.1.1 Calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ) for Piroxicam pure sample

Equation of the line y = 3988.1x + 0.3674

Table 4.13 Calculation of LOD and LOQ for Piroxicam pure sample

Concentration (x)	**Peak Area (Y)**	**Y_{est}**	**$Y - Y_{est}$**
0.006	24.21	24.2968	0.0868
0.005	19.94	20.3079	0.3679
0.004	16.82	16.3198	0.5002
0.002	8.32	8.3436	0.0236
0.001	4.95	4.3555	0.5945
0.0005	2.14	2.3615	0.2215
0.0002	0.77	1.1650	0.3950
			$\sum(Y - Y_{est}) = 2.1895$

Number of injections = 6

Degrees of freedom (n – 1) = 5

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

$= (2.1895/5)^2$

$= (0.4379)^2$

$= 0.1918$

$LOD = 3.3\sigma / S$

$= (3.3 \times 0.1918)/ 3988.1$

$= 0.000159$

LOQ = 10σ / S

= (10 x 0.1918)/ 3988.1 = 0.00048

4.6.2 Linearity

Refer to Table LPP to SSM in the appendix

4.6.3 Sensitivity

Refer to Table SSP to SSM in the appendix

4.6.4 Precision

Refer to Table PPP to PMP in the appendix

4.7 Results of stability studies on pure samples in solution

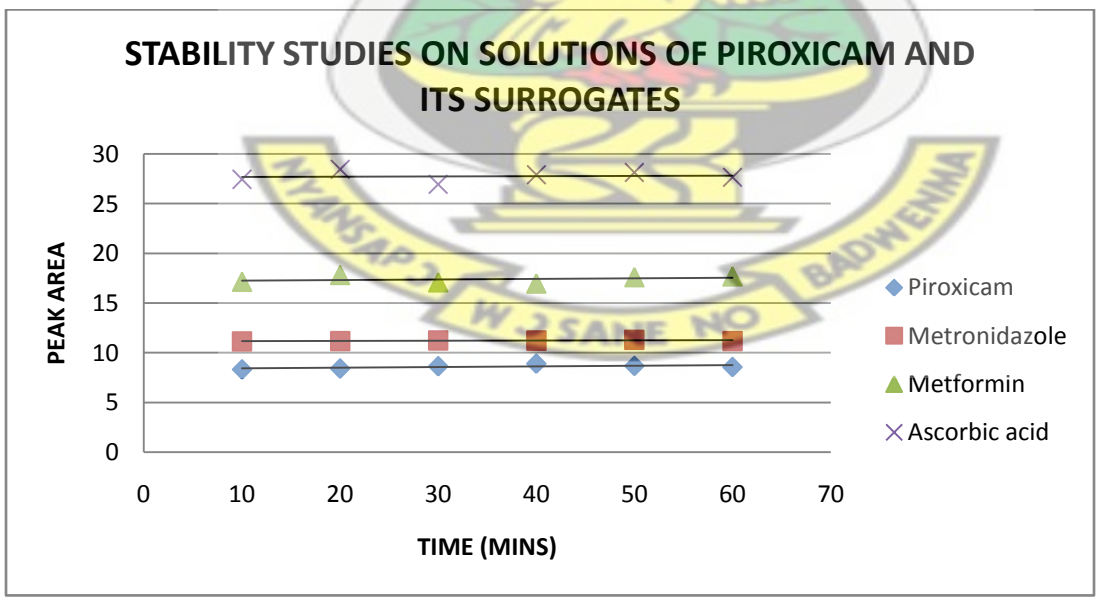

Figure 4.35 Stability studies on piroxicam and its surrogates in solution

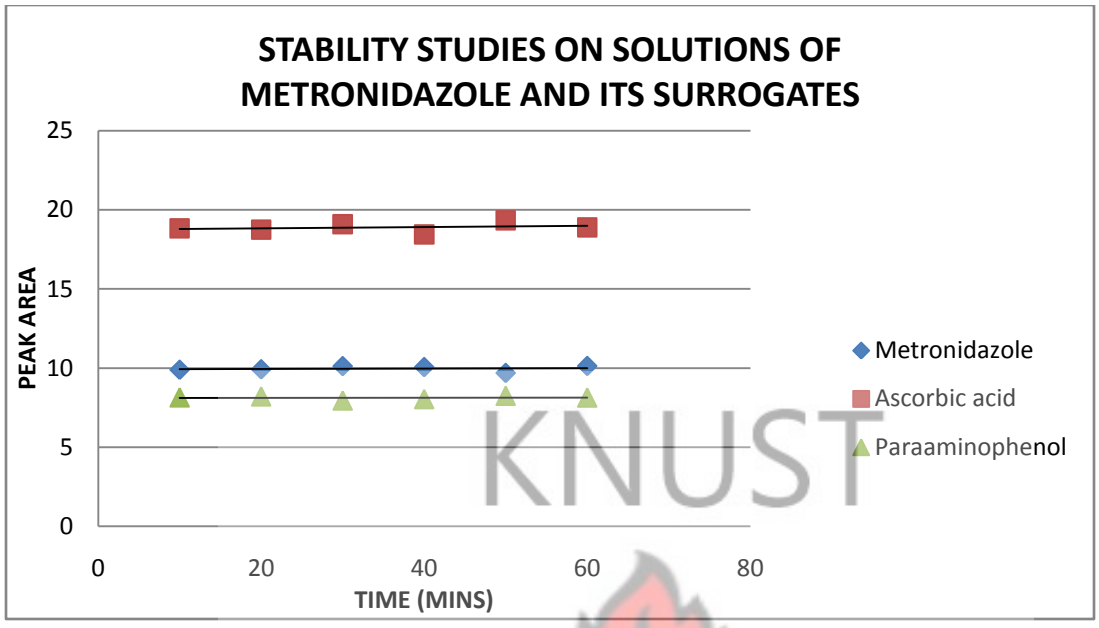

Figure 4.36 Stability studies on metronidazole and its surrogates in solution

4.8 Calculation of K value

4.8.1 Calculation of K value for Piroxicam using Metronidazole as surrogate standard

Concentration of Piroxicam, Canalyte = 0.0020%w/v

Concentration of Metronidazole, Cstandard = 0.0016%w/v

Peak area of Piroxicam, Aanalyte =5.22

Peak area of Metronidazole, Astandard = 2.14

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

= 5.22 x 0.0016 / 0.0020 x 2.14

= 1.9314

Table 4.14 K values obtained for the various surrogate reference standards in the Analysis of Piroxicam

Surrogate Standard	Calculated K value					
	1	2	3	4	5	Mean K
Metronidazole	1.9344	1.9314	1.9447	1.9556	1.9392	1.9411±0.004
Metformin	1.6266	1.6286	1.6061	1.6334	1.6051	1.6200±0.006
Ascorbic Acid	0.4675	0.4649	0.4400	0.4685	0.4435	0.4569±0.006

4.8.2 Calculation of K value for Metronidazole using Ascorbic acid as surrogate standard

Concentration of analyte = 0.01%w/v

Concentration of standard = 0.005%w/v

Peak area of analyte = 12.11

Peak area of standard = 8.93

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

= 12.11 x 0.005 / 0.01 x 8.93

= 0.6784

Table 4.15 K values obtained for the various surrogate reference standards in the Analysis of Metronidazole

Surrogate Standard	**Calculated K value**					
	1	**2**	**3**	**4**	**5**	**Mean K**
Ascorbic Acid	0.6864	0.6784	0.6674	0.6808	0.6685	0.6763±0.004
Para aminophenol	1.0928	1.1834	1.1100	1.1250	1.1030	1.1229±0.016

4.9 Calculation of percentage content of Piroxicam in Piroxicam capsules using the method developed

4.9.1 Piroxicam capsules manufactured by Ernest Chemist Ltd.

Average weight of content of capsule = 0.2630g.

0.2630g ≡ 20mg of Piroxicam;

10mg ≡ (10mg × 0.2630g) / 20mg ≡ 0.1315g

Hence, 0.1315g will contain 10mg of pure Piroxicam.

Surrogate standard = Metronidazole;

Analyte = Piroxicam.

Area of analyte, Aa = 13.26;

Area of standard, As = 7.13;

Concentration of standard, Cs = 0.005%w/v

Thus the Average K value (Metronidazole) = 1.9411

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

= (13.26 × 0.005%w/v) / (1.9411 × 7.13)

= 0.06625 / 13.84004

= 0.004787% w/v

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

= (0.004787 / 0.005) × 100%

= 95.74%

Table 4.16 Results of Mean percentage contents of Piroxicam capsules analysed with the different surrogate reference standards

Surrogate	**Mean percentage content**			
	Ernest Chemist	**Kinapharma**	**Letap**	**Luex**
Metronidazole	95.97±0.49	98.87±0.32	101.78±0.32	98.02±0.19
Metformin	94.68±0.20	96.82±0.64	102.06±0.47	98.09±0.08
Ascorbic acid	96.08±0.16	99.45±0.42	100.93±0.43	98.58±0.47

Table 4.17 Results of Mean percentage contents of Metronidazole tablets analysed with the different surrogate reference standards

Surrogate	Mean percentage content			
	Ernest Chemist	**Letap**	**M & G**	**Maxheal**
Ascorbic acid	97.84±0.31	104.15±0.19	98.03±0.35	104.14±0.47
Para aminophenol	98.44±0.25	104.31±0.20	97.48±0.36	104.29±0.37

4.10 Comparison of the Accuracy of the Method Developed with Standard Method (BP 2007) using t-Test

$t_{exp} = (X_d / S_d) \times \sqrt{N}$ [80]

Where;

X_d = the mean difference between paired values,

S_d = the estimated standard deviation of the differences and

N = number of values within the sets.

For four (4) degrees of freedom, the critical values of "t" at the 95% (P = 0.05) levels for a two-tailed test is 2.78.

4.10.1 Sample calculation for t_{exp}

The percentage content for Metronidazole in Metronidazole tablets from Ernest Chemist Ltd., using the standard method in the BP 2007 and the developed method with Ascorbic acid as the surrogate reference standard is indicated in the table below:

Table 4.18 Sample calculation for t_{exp}

Percentage content of Metronidazole Tablet (%)		
Standard method	New method	Standard method – New method
97.01	98.86	-1.85
99.02	96.91	2.11
98.97	97.54	1.43
97.70	97.95	-0.25
98.39	97.95	0.44
		$X_d = 0.376$

$X_d = 0.376$

$S_d = 1.5386$

$N = 5$

$texp = (X_d / S_d) \times \sqrt{N}$

$= (0.376/ 1.5386) \times \sqrt{5}$

$= 0.5464$

Table 4.19 t-Test for Piroxicam capsules manufactured by Ernest Chemist Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**	**New Method (Metformin as surrogate) $\%^{w}/_{w}$**	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**
95.95	96.28	95.10	95.54
96.40	95.74	93.97	96.00
94.85	96.30	94.57	96.48
96.85	96.12	95.14	96.32
96.78	95.42	94.70	96.06
	t = 0.3945	t = 3.8126	t = 0.1987

From the above table, it can be observed that the calculated t-values are smaller than the critical value of 2.78 when Metronidazole and Ascorbic acid were used as surrogate reference standards. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level. However, the calculated t-value is greater than the critical value of 2.78

when Metformin was used as surrogate reference standard. Hence, there is a significant difference between the two methods. The null hypothesis is rejected at the 95% probability level.

Table 4.20 t-Test for Piroxicam capsules manufactured by Kinapharma Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**	**New Method (Metformin as surrogate) $\%^{w}/_{w}$**	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**
99.52	98.17	95.85	98.51
100.95	99.50	98.34	99.89
99.84	99.75	95.84	98.91
98.89	98.44	98.43	99.07
98.74	98.47	95.65	100.89
	t = 2.5508	t = 4.4327	t = 0.2178

From the above table, it can be observed that the calculated t-values are smaller than the critical value of 2.78 when Metronidazole and Ascorbic acid were used as surrogate reference standards. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level. However, the calculated t-value is greater than the critical value of 2.78 when Metformin was used as surrogate reference standard. Hence, there is a significant difference between the two methods. The null hypothesis is rejected at the 95% probability level.

Table 4.21 t-Test for Piroxicam capsules manufactured by Letap Pharmaceuticals Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**	**New Method (Metformin as surrogate) $\%^{w}/_{w}$**	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**
101.12	101.66	101.20	100.44
103.01	101.06	103.3	100.06
101.12	102.56	103.04	101.75
100.98	102.46	101.02	100.21
101.45	101.14	101.76	102.19
	t = 0.3756	t = 1.4992	t = 0.9101

The calculated t-values are smaller than the critical value of 2.78. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Table 4.22 t-Test for Piroxicam capsules manufactured by Luex Pharmaceuticals Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**	**New Method (Metformin as surrogate) $\%^{w}/_{w}$**	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**
98.14	98.03	98.06	98.00
97.24	97.75	97.99	98.50
99.14	97.68	98.22	99.62
97.87	98.72	98.33	99.59
100.11	97.93	97.87	97.19
	t = 0.8236	t = 0.7526	t = 0.09814

The calculated t-values are smaller than the critical value of 2.78. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Table 4.23 t-Test for Metronidazole tablets manufactured by Ernest Chemist Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**	**New Method (Para aminophenol as surrogate) $\%^{w}/_{w}$**
97.01	98.86	98.55
99.02	96.91	98.40
98.97	97.54	99.06
97.70	97.95	98.68
98.39	97.95	97.53
	t = 0.5464	t= 0.4804

The calculated t-values are smaller than the critical value of 2.78. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Table 4.24 t-Test for Metronidazole tablets manufactured by Letap Pharmaceuticals Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**	**New Method (Para aminophenol as surrogate) $\%^{w}/_{w}$**
103.33	104.69	103.86
103.81	103.93	103.85
103.70	103.61	104.54
104.98	104.05	104.38
104.93	104.48	104.90
	t = 0.0052	t = 0.6298

The calculated t-values are smaller than the critical value of 2.78. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Table 4.25 t-Test for Metronidazole tablets manufactured by M & G Pharmaceuticals Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**	**New Method (Para aminophenol as surrogate) $\%^{w}/_{w}$**
97.29	97.48	97.43
98.58	97.10	96.63
95.96	98.66	97.62
97.93	97.93	96.95
96.58	98.97	98.76
	t = 0.9675	t = 0.2703

The calculated t-values are smaller than the critical value of 2.78. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Table 4.26 t-Test for Metronidazole tablets manufactured by Maxheal Laboratories Ltd

Standard Method (B.P., 2007) $\%^{w}/_{w}$	**New Method (Ascorbic acid as surrogate) $\%^{w}/_{w}$**	**New Method (Para aminophenol as surrogate) $\%^{w}/_{w}$**
104.38	104.50	104.16
105.29	105.56	104.81
105.85	104.08	105.22
104.16	103.92	104.25
104.41	102.62	103.02
	t = 1.4986	t = 2.1182

4.11 Results of Relative Standard Deviation (RSD) and Standard Error of the Mean (SEM)

Table 4.27 RSD and SEM of Piroxicam capsules assayed using the B.P. 2007

Name of Manufacturer	Mean	Standard Deviation	RSD %	SEM
Ernest Chemist Ltd	96.16	0.8182	0.8509	0.3659
Kinapharma Ltd	99.59	0.8845	0.8881	0.3956
Letap Pharmaceuticals	101.34	1.0568	1.0428	0.4726
Luex Pharmaceuticals	98.50	1.1309	1.1481	0.5058

Table 4.28 RSD and SEM of Metronidazole tablets assayed using the USP 2007

Name of Manufacturer	Mean	Standard Deviation	RSD %	SEM
Ernest Chemist Ltd	98.08	0.7626	0.7775	0.3410
Letap Pharmaceuticals	104.15	0.7563	0.7262	0.3382
M & G Pharmaceuticals	97.44	1.1184	1.1478	0.5592
Maxheal Laboratories Ltd	104.84	0.7210	0.6877	0.3224

Table 4.29 RSD and SEM of Piroxicam capsules manufactured by Ernest Chemist LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Metronidazole	95.97	0.3817	0.3977	0.1707
Metformin	94.67	0.4575	0.4833	0.2046
Ascorbic acid	96.08	0.3592	0.3739	0.1606

Table 4.30 RSD and SEM of Piroxicam capsules manufactured by Kinapharma LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Metronidazole	98.87	0.7082	0.7163	0.3167
Metformin	96.82	1.4393	1.4866	0.6392
Ascorbic acid	99.65	0.9081	0.9113	0.4061

Table 4.31 RSD and SEM of Piroxicam capsules manufactured by Letap Pharmaceuticals LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Metronidazole	101.78	0.7094	0.6970	0.3173
Metformin	102.06	1.0499	1.0287	0.4695
Ascorbic acid	100.93	0.9715	0.9625	0.4345

Table 4.32 RSD and SEM of Piroxicam capsules manufactured by Luex Pharmaceuticals LTD., India assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Metronidazole	98.06	0.3905	0.3982	0.1746
Metformin	98.09	0.1828	0.1864	0.0818
Ascorbic acid	98.58	1.0460	1.0611	0.4678

Table 4.33 RSD and SEM of Metronidazole tablets manufactured by Ernest Chemist LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Ascorbic acid	97.84	0.7105	0.7262	0.3178
Paraaminophenol	98.44	0.5665	0.5755	0.2534

Table 4.34 RSD and SEM of Metronidazole tablets manufactured by Letap Pharmaceuticals LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Ascorbic acid	104.15	0.4332	0.4159	0.1937
Paraaminophenol	104.31	0.4527	0.4340	0.2025

Table 4.35 RSD and SEM of Metronidazole tablets manufactured by M & G Pharmaceuticals LTD., Ghana assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Ascorbic acid	97.99	0.7952	0.8115	0.3556
Paraaminophenol	97.49	0.8161	0.8371	0.3650

Table 4.36 RSD and SEM of Metronidazole tablets manufactured by Maxheal Laboratories LTD., India assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Ascorbic acid	104.14	1.0616	1.0194	0.4748
Paraaminophenol	104.29	0.8317	0.7975	0.3719

4.12 Relative Precision of the New Method to the Standard Method

Null Hypothesis: There are no significant differences between the precisions of the two methods at the 95% probability level.

The standard method and the developed method were subjected to the F-test to determine whether their sets of data differ in precision; a two-sided test.

$F = S_1^2 / S_2^2$ [81]

Where:

S^2 is the variance, with the largest variance value being the numerator so that $F > 1$.

Sample size, n of the standard method = 5; number of degrees of freedom = 4

Sample size, n of the new method = 5; number of degrees of freedom = 4

Critical value of F at P = 0.05 (95%) level = 9.605

Manufacturer: Ernest Chemist LTD.

Mean of Standard method, B.P. 2007 = 96.16

Standard deviation, S of Standard method, B.P. 2007 = 0.8182

Therefore variance, S^2 of Standard method, B.P. 2007 = 0.6695

Table 4.37 Piroxicam capsules manufactured by Ernest Chemist LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F value
Metronidazole	95.97	0.3817	0.1457	4.5951
Metformin	94.67	0.4575	0.2093	3.1988
Ascorbic acid	96.08	0.3592	0.1290	5.1899

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Kinapharma LTD.

Mean of Standard method, B.P. 2007 = 99.59

Standard deviation, S of Standard method, B.P. 2007 = 0.8845

Therefore variance, S^2 of Standard method, B.P. 2007 = 0.7823

Table 4.38 Piroxicam capsules manufactured by Kinapharma LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F value
Metronidazole	98.87	0.7082	0.5015	1.5599
Metformin	96.82	1.4393	2.0716	2.6481
Ascorbic acid	99.65	0.9081	0.8246	1.0541

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Letap Pharmaceuticals LTD.

Mean of Standard method, B.P. 2007 = 101.34

Standard deviation, S of Standard method, B.P. 2007 = 1.0568

Therefore variance, S^2 of Standard method, B.P. 2007 = 1.1168

Table 4.39 Piroxicam capsules manufactured by Letap Pharmaceuticals LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Metronidazole	101.78	0.7094	0.5032	2.2194
Metformin	102.06	1.0499	1.1023	1.0132
Ascorbic acid	100.93	0.9715	0.9438	1.1833

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Luex Pharmaceuticals LTD.

Mean of Standard method, B.P. 2007 = 98.50

Standard deviation, S of Standard method, B.P. 2007 = 1.1309

Therefore variance, S^2 of Standard method, B.P. 2007 = 1.2789

Table 4.40 Piroxicam capsules manufactured by Luex Pharmaceuticals LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Metronidazole	98.06	0.3905	0.1525	8.3862
Metformin	98.09	0.1828	0.0334	38.2904
Ascorbic acid	98.58	1.0460	1.0941	1.1689

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Ernest Chemist LTD.

Mean of Standard method, B.P. 2007 = 98.08

Standard deviation, S of Standard method, B.P. 2007 = 0.7626

Therefore variance, S^2 of Standard method, B.P. 2007 = 0.5816

Table 4.41 Metronidazole tablets manufactured by Ernest Chemist LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Ascorbic acid	97.84	0.7105	0.5048	1.1521
Paraaminophenol	98.44	0.5665	0.3209	1.8124

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Letap Pharmaceuticals LTD.

Mean of Standard method, U.S.P. 2007 = 104.15

Standard deviation, S of Standard method, U.S.P. 2007 = 0.7563

Therefore variance, S^2 of Standard method, U.S.P. 2007 = 0.5720

Table 4.42 Metronidazole tablets manufactured by Letap Pharmaceuticals LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Ascorbic acid	104.15	0.4332	0.1877	3.0474
Paraaminophenol	104.31	0.4527	0.2049	2.7916

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: M & G Pharmaceuticals LTD.

Mean of Standard method, U.S.P. 2007 = 97.44

Standard deviation, S of Standard method, U.S.P. 2007 = 1.1184

Therefore variance, S^2 of Standard method, U.S.P. 2007 = 1.2505

Table 4.43 Metronidazole tablets manufactured by M & G Pharmaceuticals LTD.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Ascorbic acid	97.99	0.7952	0.6323	1.9777
Paraaminophenol	97.49	0.8161	0.6660	1.8776

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Manufacturer: Maxheal Laboratories LTD.

Mean of Standard method, U.S.P. 2007 = 104.84

Standard deviation, S of Standard method, U.S.P. 2007 = 0.7210

Therefore variance, S^2 of Standard method, U.S.P. 2007 = 0.5198

Table 4.44 Metronidazole tablets manufactured by Maxheal Laboratories LTD.

Surrogate Reference Standard	**Mean**	**Standard Deviation**	**Variance**	**F-test**
Ascorbic acid	104.14	1.0616	1.1270	2.1681
Paraaminophenol	104.29	0.8317	0.6917	1.3308

The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Identification test

All the pure samples used in this thesis were carried through a series of identification test such as melting point and assay to ensure their true identity.

5.1.1.1 Piroxicam

The assay of Piroxicam yielded an average percentage purity of 99.61%. The percentage purity stated in the British Pharmacopoeia ranges from 98.5% to 101%. The experimental melting point range was from 241^{o}C to 244^{o}C. The British Pharmacopoeia melting point range for Piroxicam is 240 ^{o}C to 245 ^{o}C. The sample analysed is therefore Piroxicam.

5.1.1.2 Metronidazole

The assay of Metronidazole yielded an average percentage purity of 100.49%. The percentage purity stated in the British Pharmacopoeia ranges from 99% to 101%. The experimental melting point range was from 161^{o}C to 163^{o}C. The British Pharmacopoeia melting point range for Metronidazole is 159^{o}C to 163^{o}C. The sample analysed is therefore Metronidazole.

5.1.1.3 Metformin

The assay of Metformin yielded an average percentage purity of 100.32%. The percentage purity stated in the British Pharmacopoeia ranges from 98.5% to 101%. The experimental melting point range was from 222 ^{o}C to 224^{o}C. The British Pharmacopoeia melting point range for Metformin is 222^{o}C to 226^{o}C. The sample analysed is therefore Metformin.

5.1.1.4 Ascorbic acid

The assay of Ascorbic acid yielded an average percentage purity of 100.00%. The percentage purity stated in the British Pharmacopoeia ranges from 99% to 100.5%. The experimental melting point range was from 190 ^{o}C to 191 ^{o}C. The British Pharmacopoeia melting point range for Ascorbic acid is 190 ^{o}C to 192 ^{o}C. The sample analyzed is therefore Ascorbic acid.

5.1.1.5 Paraaminophenol

The experimental melting point range was from 185 to 188 ^{o}C. The British Pharmacopoeia melting point is stated as about 186 ^{o}C for Paraaminophenol. The sample analyzed is therefore Paraaminophenol.

5.1.2 Uniformity of weight

5.1.2.1 Piroxicam

The uniformity of weight test carried out on capsules from Ernest Chemist Limited, Ghana yielded an average weight of 263.0mg The average weights for capsules from Kinapharma Limited, Ghana, Letap Pharmaceuticals Limited, Ghana and Luex Pharmaceutical Limited, India were found to be 277.2mg, 202.7mg and 191.4mg respectively when uniformity of weight test were performed on them.

Percentage deviations were calculated for each capsule and not more than 2 of the individual masses deviated from the average mass by more than the percentage deviation shown in Table 5.1 below and none deviated by more than twice that percentage as stated in the British Pharmacopoeia.

Table 5.1 Uniformity of weight of capsules

Average weight of capsule	Percentage Deviation allowed	Number of capsules
Less than 300mg	±10	20
300mg or more	±7.5	20

Source: BP 2007

All the brands of capsules used had an average weight of less than 300mg. A look at tables U1 to U4 shows that none of the capsules recorded a percentage deviation greater than 10. It can therefore be inferred that the batch of piroxicam capsules which were taken through the uniformity of weight test are therefore within the control limits of pharmacopoeial standards.

5.1.2.2 Metronidazole

The uniformity of weight test carried out on tablets from Ernest Chemist Limited, Ghana yielded an average weight of 591.3mg. The average weights for tablets from Letap Pharmaceuticals Limited, Ghana, M & G Pharmaceuticals Limited, Ghana and Maxheal Laboratories Ltd were found to be 502.0mg, 506.4mg and 639.6mg respectively when uniformity of weight test were performed on them.

Percentage deviations were calculated for each tablet and not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation shown in Table 5.2 below and none deviated by more than twice that percentage as stated in the British Pharmacopoeia.

Table 5.2 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

All the brands of tablets used had an average weight of more than 250mg. A look at tables U5 to U8 shows that none of the tablets recorded a percentage deviation greater than 5. It can therefore be inferred that the batch of metronidazole tablets which were taken through the uniformity of weight test are therefore within the control limits of pharmacopoeial standards.

5.1.3 Stability studies on drug samples in solution

For the stability test, the same sample solution was injected six times at an interval of 10 minutes for one hour. A graph of peak area against time plotted showed a linear plot parallel to the X axis for all the pure drug samples used suggesting that it is safe to analyze the sample within one hour.

5.1.4 HPLC Method Development

The goal of this study was to develop a rapid, accurate, precise reliable least time consuming HPLC method for the analysis of Piroxicam in Piroxicam capsules in the presence of surrogate reference standards, using the most commonly employed C-18 column with UV detector.

Samples containing ionizable compounds are strongly influenced by pH of the mobile phase. For acids, the retention time decreases as the pH of the mobile phase is increased. Greater charge can

be thought of as an extreme case of polarity. At pH's above the analyte's pKa, the acidic analyte carries a negative charge and behaves as an extremely polar molecule. In order to achieve adequate retention, the mobile phase should be highly aqueous. Below its pKa, the acidic analyte is neutral and much more hydrophobic.

When the acid standards were introduced to unbuffered, neutral mobile phase of 10% methanol, poor peak shape resulted. This result can be traced to a mismatch caused by the acidic nature of the sample and zero buffer strength in the neutral mobile phase. The sample therefore experiences a pH gradient during the first part of the separation, which usually causes ionizable compounds to exhibit broad peak shape and poor retention reproducibility. Addition of a phosphate buffer eliminated the broad tailing peaks and created rugged conditions suitable for successful assay.

Chemical structure and chemical properties are the most important facts that predict chromatographic behavior. In this investigation, the best separation of Piroxicam and its surrogate was achieved using an ODS C 18 Phenomenex 250 x 4.6mm column. The best results were obtained using mobile phase 50:50 methanol: phosphate buffer pH 6.2. The lower percentage of methanol initially used in mobile phase resulted in peak tailing of the components and long analysis duration while higher percentage of methanol in mobile phase resulted in very little analysis duration. Optimal retention times (piroxicam – 6.83±0.065minutes, Metronidazole – 4.07±0.058 minutes, Metformin – 3.42±0.049 minutes and Ascorbic acid – 2.56±0.055 minutes) were achieved when the pH of mobile phase was adjusted to 6.2. Small changes in pH of the mobile phase had a great influence to the chromatographic behavior of these substances.

The best separation of Metronidazole and its surrogate was achieved using an ODS C 18 Phenomenex 250 x 4.6mm column. The best results were obtained using mobile phase 70:30

water: methanol. The retention times obtained for Metronidazole, Ascorbic acid and Para aminophenol were 4.24±0.014, 1.80±0.012 and 2.82±0.015 minutes respectively.

5.1.5. Analytical Performance Parameters

5.1.5.1 Limit of Detection (LOD)

LOD is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The Limit of Detection for the analysis of Piroxicam capsules was determined to be 0.000159, 0.000077, 0.000074 and 0.000064%w/v for Piroxicam, Metronidazole, Metformin and Ascorbic acid respectively. The Limit of Deteection for the analysis of Metronidazole tablets was determined to be 0.0000185, 0.000334 and 0.0000295 for Metronidazole, Ascorbic acid and Para aminophenol respectively. This was carried out prior to the analysis to determine the smallest amount of the analyte and the surrogate reference standard that could be detected using the new method. This information informed the choice of the concentrations used for the analysis. All the selected concentrations were above those obtained so as to be sure of the sample that the samples would be detected at the chosen concentrations.

5.1.5.2 Limit of Quantitation (LOQ)

LOQ is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The Limit of Quantitation for the analysis of Piroxicam capsules was determined to be 0.00048, 0.00023, 0.00022 and 0.00019%w/v for Piroxicam, Metronidazole, Metformin and Ascorbic acid respectively. The Limit of Quantitation for the analysis of Metronidazole tablets was determined to be 0.0000560, 0.001032 and 0.0000891 for Metronidazole, Ascorbic acid and Para aminophenol respectively. This test was also carried out

prior to the analysis to determine the lowest level of an analyte that can be quantified with some degree of certainty.

5.1.5.3 Linearity

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. Linearity is determined by calculating the regression line using a mathematical treatment of the results versus analyte concentration. A correlation coefficient of ≥ 0.99 is regarded as indicating linearity. The results, as shown in Tables LPP to LMP in the appendix show an appreciable level of linearity.

5.1.5.4 Specificity

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/or impurities. Determination of this can be carried out by assessing the peak identity and purity.

These parameters were determined by comparing the chromatograms of the Piroxicam pure sample with that of the Piroxicam capsules. The chromatograms of Metronidazole pure sample and Metronidazole tablets were also compared. The chromatogram of the Prioxicam pure sample and Piroxicam capsules presented a peak with mean retention time of 6.83±0.065 and 6.84± 0.016 minutes respectively as shown in table SSP in the appendix.

The chromatogram of the Metronidazole pure sample and Metronidazole tablets presented a peak in mean retention time of 4.24±0.014 and 4.26±0.006 (min) respectively as shown in table SSM on the appendix.

Also when Piroxicam pure sample and its surrogates (Metronidazole, Metformin and Ascorbic acid) were injected together, the chromatogram peaks were well resolved as shown in Fig 27, indicating the high specificity of the method.

The retention times are good values for routine procedures in quality control.

5.1.5.5 Relative Standard Deviation, precision and repeatability

For an HPLC system this would involve the reproducibility of a number of replicate injections (ie 6) of an analytical solution.

The USP requires that unless otherwise specified by a method:

- if a relative standard deviation of <2% is required then five replicate injections should be used
- if a relative standard deviation of >2% is required then six replicate injections should be used

The analysis method repeatability was examined by the injection of six different sample solutions, which were prepared using the same sample preparation procedure. The results as shown in tables PPP to PMP in the appendix showed good repeatability since all the Relative Standard Deviations obtained for different concentrations of both the analytes and the surrogate reference standard were less than 2.

5.1.6. Determination of the Constant K

The concentration of the both the analyte and the surrogate reference standards and their respective peak areas were used to calculate the respective K value using the formula for the

determination of K. From the results obtained, it was observed that for each of the surrogate reference standards that, a change in concentration or other wise of the analyte has no significant effect on the K value.

Different K values were obtained when each of the surrogate reference standards were used for the analysis. Unsaturated groups such as C = O and C = C, and auxochromes such as OH, NH_2 and OCH_3 present in the compounds contribute to various degrees of UV absorption, and may account for the varying K constants.

5.1.7. Determination of Percentage content using the constant K

Various brands of Piroxicam capsules and Metronidazole tablets were assayed to ascertain the content of the active ingredients using the calculated values and a surrogate reference standard in each case. The results were recorded in tables PPE 1 to PMX 2 in the appendix. The range of percentage content stated in the British Pharmacopoeia 2007 (BP) for Piroxicam capsules and the United States Pharmacopoeia 2007 (USP) for Metronidazole tablets is from 95% to 105% and 90% to 110% respectively. From the table 4.16 and 4.17 in chapter four, it could be observed that almost all the calculated percentage content fell within the range stated in the BP and USP for Piroxicam capsules and Metronidazole tablets respectively. The percentage contents of the standard method and the new method were comparable.

5.1.8 Determination of Percentage Content of Piroxicam in Piroxicam capsules Using Standard Method in the British Pharmacopoeia (2007)

Piroxicam capsules from four (4) different manufacturers namely Ernest Chemist Limited, Ghana, Kinapharma Limited, Ghana, Letap Pharmaceuticals Limited, Ghana and Luex Pharmaceuticals Limited, UK were analysed using the standard method stated in the British

Pharmacopoeia (BP) 2007 for Piroxicam capsules. The average percentage contents obtained for the analysis of the brands from Ernest Chemist Limited, Ghana, Kinapharma Limited, Ghana, Letap Pharmaceuticals Limited, Ghana and Luex Pharmaceuticals Limited, China were 96.17, 99.59, 101.34 and 98.50% respectively. The BP stipulates that Piroxicam capsules should have a percentage content range of 95 to 105%. All the brands analyzed fell within this range and hence passed the test.

5.1.9 Determination of Percentage Content of Metronidazole in Metronidazole tablets Using Standard Method in the United States Pharmacopoeia (2007)

Metronidazole tablets from four (4) different manufacturers namely Ernest Chemist Limited, Ghana, Letap Pharmaceuticals Limited, Ghana, M & G Pharmaceuticals Limited, Ghana and Maxheal Laboratories Limited, India were analysed using the standard method stated in the United States Pharmacopoeia (USP) 2007. The average percentage contents obtained for the analysis of the brands from Ernest Chemist Limited, Ghana, Letap Pharmaceuticals Limited, Ghana, M & G Pharmaceuticals Limited, Ghana and Maxheal Laboratories Limited, India were 98.08, 104.15, 97.44 and 104.82 respectively. The USP stipulates that Metronidazole tablets should have a percentage content range of 90 to 110%. All the brands analyzed fell within this range and hence passed the test.

5.1.10. Comparison of the Accuracy of Developed Method to that of Standard Method using T-test

5.1.10.1 Piroxicam capsules

The T-test is used to compare the experimental means of two sets of data or to compare the experimental mean of one set of data with a known or reference value.

The method used tests whether there is a significant difference between the experimental result and the values for the standard, regardless of the sign of the difference. In this situation there was no idea, prior to the experiment, as to whether any difference between the experimental mean and the reference value will be positive or negative. Thus the test used covered either possibility and hence, a two-sided (or two-tailed) was employed.

The Null Hypothesis states that the means of the two methods do not differ significantly at the 95% probability level.

For four (4) degrees of freedom, the critical values of "t" at the 95% (P = 0.05) levels for a two-tailed test is 2.78. [80]

The t_{exp} of Piroxicam capsules manufactured by Ernest Chemist were 0.3945, 3.8126 and 0.1987 when Metronidazole, Metformin and Ascorbic Acid were used as surrogate reference standards respectively.

Piroxicam capsules manufactured by Kinapharma Ltd had the following t_{exp} values; 2.5508, 4.4327 and 0.2178 when Metronidazole, Metformin and Ascorbic Acid were used as surrogate reference standards respectively.

Piroxicam capsules manufactured by Letap Pharmaceuticals had the following t_{exp} values; 0.3756, 1.4992 and 0.9101 when Metronidazole, Metformin and Ascorbic Acid were used as surrogate reference standards respectively.

Also, the t_{exp} of Piroxicam manufactured by Luex Pharmaceuticals were 0.8236, 0.7526 and 0.0981 when Metronidazole, Metformin and Ascorbic Acid were used as surrogate reference standards respectively.

From the above, the t_{exp} values obtained when Metronidazole and Ascorbic acid were used as surrogate reference standards are less than the t at 95% probability levels and hence there was no significant difference between the means of the standard method (BP 2007) and the method developed.

When Metformin was used as a surrogate reference standard for the analysis of Piroxicam capsules from Ernest Chemist and Kinapharma Ltd, the t_{exp} values obtained (3.8126 and 4.4327 respectively) were greater than t at 95% probability level hence there was a significant difference between the accuracy of the two methods. The assay results of the Piroxicam capsules from Ernest Chemist and Kinapharma Limited fell within the range stated in the British Pharmacopoeia (BP) so also were the results of the standard method.

5.1.10.2 Metronidazole tablets

The t_{exp} of Metronidazole tablets manufactured by Ernest Chemist were 0.5464 and 0.4804 when Ascorbic Acid and Para aminophenol were used as surrogate reference standards respectively.

Metronidazole tablets manufactured by Letap Pharmaceuticals had the following t_{exp} values; 0.0052 and 0.6298 when Ascorbic Acid and Para aminophenol were used as surrogate reference standards respectively.

Metronidazole tablets manufactured by M & G Pharmaceuticals had the following t_{exp} values; 0.9675 and 0.2703 Ascorbic Acid and Para aminophenol were used as surrogate reference standards respectively.

Also, the t_{exp} of Metronidazole tablets manufactured by Luex Pharmaceuticals were 1.4986 and 2.1182 when Ascorbic Acid and Para aminophenol were used as surrogate reference standards respectively.

From the above, all the texp are less than the t at 95% probability levels for all tablets and hence there was no significant difference between the means of the standard method (USP 2007) and the method developed. The null hypothesis is therefore accepted at the 95% probability level.

5.1.11 Relative Precision of the New method to the Standard method for the Assay of Piroxicam capsules and Metronidazole tablets using the F-test.

This test was carried out to ascertain whether the two methods ie the standard method and the developed method differ in their precision. It was to test whether two standard deviations differ significantly and a two-sided (two tailed) test was appropriate. The *F*-test considers the ratio of the two sample variances, i.e. the ratio of the squares of the standard deviations.

F-test for testing equality of variance is used to test the hypothesis of equality of two population variances. The Null Hypothesis states that there are no significant differences between the precisions of the two methods at the 95% probability level.

The critical value of F at the probability level of 95% level is 9.605. [81] The calculated F-test value of Piroxicam capsules manufactured by Ernest Chemist Ltd, Ghana, using Metronidazole, Metformin and Ascorbic acid as the surrogate reference standard against the standard method (B.P. 2007) were 4.5951, 3.1988 and 5.1899 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the precisions of the two methods. The null hypothesis is accepted at the 95% probability level.

Piroxicam capsules manufactured by Kinapharma Ltd, Ghana, using Metronidazole, Metformin, and Ascorbic acid as the surrogate reference standard against the standard method (B.P. 2007) were 1.5599, 2.6481 and 1.0541 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Piroxicam capsules manufactured by Letap Pharmaceuticals Ltd., Ghana, using Metronidazole, Metformin and Ascorbic acid as the surrogate reference standard against the standard method (B.P. 2007) were 2.2194, 1.0132 and 1.1833 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Piroxicam capsules manufactured by Luex Pharmaceuticals Ltd., Ghana, using Metronidazole, Metformin and Ascorbic acid as the surrogate reference standard against the standard method (B.P. 2007) were 8.3862, 38.2904 and 1.1689 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Metronidazole tablets manufactured by Ernest Chemist Ltd., Ghana, using Ascorbic acid and Para aminophenol as the surrogate reference standard against the standard method (U.S.P. 2007) were 1.1521 and 1.8124 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Metronidazole tablets manufactured by Letap Pharmaceuticals Ltd., Ghana, using Ascorbic acid and Para aminophenol as the surrogate reference standard against the standard method (U.S.P. 2007) were 3.0474 and 2.7916 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Metronidazole tablets manufactured by M & G Pharmaceuticals Ltd., Ghana, using Ascorbic acid and Para aminophenol as the surrogate reference standard against the standard method (U.S.P. 2007) were 1.9777 and 1.8776 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

Metronidazole tablets manufactured by Maxheal Laboratories Ltd., Ghana, using Ascorbic acid and Para aminophenol as the surrogate reference standard against the standard method (U.S.P. 2007) were 2.1681 and 1.3308 respectively. The calculated F-values are less than the critical value of 9.605. Hence, there is no significant difference between the two methods. The null hypothesis is accepted at the 95% probability level.

5.2 Conclusion

An optimal Reverse Phase High Performance Liquid Chromatographic method for the determination of Piroxicam and Metronidazole in Piroxicam capsules and Metronidazole tablets respectively has been established using K calculated from the use of surrogate reference standards. The assay is simple, reproducible, sensitive, rapid and reliable and has been fully validated. All the validation procedures confirm that this developed method is suitable for the analysis of Piroxicam capsules and Metronidazole tablets.

The assay results for both the standard methods in the Bristish Pharmacopoeia and the United States Pharmacopoeia for Piroxicam Capsules and Metronidazole tablets respectively and the method developed fell within the limit stated in the respective Pharmacopoeias.

Statistically, there was a significant difference between the accuracy of the standard method and the method developed when metformin was used a surrogate reference standard in the analysis of Piroxicam from Ernest Chemist and Kinapharma Limited. However there was no significant difference between the accuaracies of the two methods when Metronidazole and Ascorbic acid were used as surrogate reference standards in the analysis of Piroxicam. Similarly, there was no significant difference between the accuracies of the two methods when Para aminophenol and Ascorbic acid were used as surrogate reference standards in the analysis of Metronidazole. Therefore Metformin may not be a good surrogate reference standard in the analysis of Piroxicam. There was no significant difference between the precisions of the two methods.

A mobile phase system found suitable for Piroxicam and its surrogates was 50:50 methanol: phosphate buffer pH 6.2. The K values obtained when Metronidazole, Metformin and Ascorbic acid were used as surrogate reference standards were 1.9411±0.004, 1.6200±0.006 and 0.4569±0.006 respectively.

A mobile phase system found suitable for Metronidazole and its surrogates was 70:30 water: methanol. The K values obtained when Ascorbic acid and Para aminophenol were used as surrogate reference standards were 0.6763±0.004 and 1.1229±0.016 respectively.

5.3 Recommendations

Surrogate reference standards must be found for the analysis of other pharmaceutical preparations to ease the burden on manufacturing companies especially in developing countries like Ghana as reference standards are becoming expensive.

Secondly, further studies should be undertaken to investigate whether variations in the wavelength will have a significant effect on the value of K.

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APPENDIX

P.1.0 Preparation of solutions

P.1.0.1 Preparation of 0.1M Perchloric Acid

Glacial acetic acid (900 mL) was measured into a 1L volumetric flask. Perchloric acid (10.2 mL of 60%) was slowly added with continuous and efficient mixing. The perchloric acid was well diluted with the glacial acetic acid before the addition of 30 mL acetic anhydride. This was done to prevent the danger of forming acetyl perchlorate. The addition was made slowly with continuous and efficient mixing. The volume was adjusted to 1 L with glacial acetic acid. The solution was allowed to stand for 24 hrs before it was used. The acetic anhydride used was to react with any molecules of water in the perchloric acid and glacial acetic acid to make the solution virtually anhydrous.

P.1.0.2 Preparation of 0.1M H_2SO_4

Specific gravity of H_2SO_4 = 1.835g/mL

98.05g H_2SO_4 in 1000mL ≡ 1M H_2SO_4

0.4904g/mol in 100mL ≡ 0.05MH_2SO_4

Percentage purity of H_2SO_4 = 98%

98% = 0.4904g/mol

100% ≡ 0.5g/mol

But volume, V = mass/specific gravity

Therefore V = 0.5g/1.835g/mL = 0.3mL

Hence 0.3mL of the stock solution of H_2SO_4 was taken and diluted to the 100mL mark with distilled water.

P.1.0.3 Preparation of 0.05M Iodine

253.8g/mol of Iodine in 1000mL ≡ 0.05M I

1.27g/mol of Iodine in 100mL ≡ 0.05M I

Percentage purity of I = 99.0%

Hence 100% = 1.28g/mol

Therefore 1.28g of I was weighed and dissolved in about 60mL of distilled water in a 100mL and swirled vigorously and made up to the mark. This is equal to 0.05M I solution.

P.1.0.4 Preparation of 0.1M $Na_2S_2O_3$

248g/mol of $Na_2S_2O_3$ in 1000mL ≡ 1M $Na_2S_2O_3$

2.48g/mol of $Na_2S_2O_3$ in 100mL ≡ 0.1M $Na_2S_2O_3$

Percentage purity of $Na_2S_2O_3$ = 98.0%

Hence 100% = 2.5g/mol

Therefore 2.5g of $Na_2S_2O_3$ was weighed and dissolved in about 60mL of distilled water in a 100mL and swirled vigorously and made up to the mark. This is equal to 0.1M $Na_2S_2O_3$ solution.

Preparation of 0.01M methanolic Hydrochloric acid

36.5g HCl in 1000mL ≡ 1M HCl

3.65g in 100mL ≡ 1M HCl

1.825g in 500mL ≡ 0.01M HCl

Percentage purity of HCl = 36%

36% = 1.825g

$$100\% = \frac{100 \times 1.825}{36}$$

$$= 5.07g$$

Specific gravity of HCl = 1.18

$$\text{Volume of HCl} = \frac{\text{mass}}{\text{Specific gravity}}$$

$$= \frac{5.07}{1.18}$$

$$= 4.30mL$$

4.30mL of HCl was measured into a 500mL volumetric flask and top up to the mark with methanol.

Titration Tables

Table T.A.P. Assay of Pure Piroxicam

Sample	A	B	C	Blank
Weight taken	0.2503	0.2500	0.2501	
Endpoint	7.70	7.60	7.70	0.10

Table T.A.M Assay of Pure Metronidazole

Sample	A	B	C	Blank
Weight taken	0.1502	0.1503	0.1502	
Endpoint	8.90	9.00	9.00	0.10

Table T.A.F. Assay of Pure Metformin

Titration	A	B	Blank
Weight taken	0.1005	0.1004	
Endpoint	6.30	6.30	0.20

Table T.A.A. Assay of Pure Ascorbic acid

Sample	A	B	C	Blank
Weight taken	0.1504	0.1502	0.1501	
Endpoint	17.50	17.50	17.40	0.10

A.1.0.1 Assay of Metformin pure sample

Titre value = 6.30mL – Blank (0.2mL) = 6.10mL

Factor of perchloric Acid = 0.9931

Actual titre = 6.9 x 0.9931

= 6.0579mL

1 mL of 0.1 M perchloric acid is equivalent to 0.01656g of $C_4H_{12}ClN_5$.

Actual amount of Metformin = 6.0579 x 0.01656g

= 0.100319g

Thus the percentage purity (%) = (0.100319/0.10) x 100 = 100.32%

Similar calculations were done to obtain percentage purity 100.32 for sample B.

Therefore average percentage purity is the calculated as follows;

Average percentage purity = (100.32 + 100.32)/ 2

= 100.32%

A.1.0.2. Assay of Metronidazole pure sample

Titre value = 8.9mL – Blank (0.1mL) = 8.80mL

Factor of perchloric Acid = 0.9931

Actual titre = 8.80 x 0.9931

= 8.7393mL

1 mL of 0.1 M perchloric acid is equivalent to 0.01712g of $C_6H_9N_3O_3$

Actual amount of metronidazole = 8.7393 x 0.01712g

= 0.1496g

Thus the percentage purity (%) = (0.1496/0.15) x 100

= 99.74%

Similar calculations were done to obtain percentage purities 100.87 and 100.87 for sample B and C respectively.

Therefore average percentage purities is the calculated as follows;

Average percentage purities = (99.74 + 100.87+ 100.87)/ 3

= 100.49%

A.1.0.3. Assay of Ascorbic acid pure sample

Titre value = 17.5mL – Blank (0.1mL) = 17.4mL

Factor of Iodine = 0.9804

Actual titre = 17.4mL x 0.9804

= 17.05896mL

1 mL of 0.05 M iodine is equivalent to 0.00881 mg of $C_6H_8O_6$

Actual amount of Ascorbic Acid = 17.05896 x 0.00881g

= 0.15029g

Percentage purity = (0.15029/0.15) x 100

= 100.19%

Similar calculations were done to obtain percentage purities 100.19 and 99.62 for sample B and C respectively.

Therefore average percentage purity is the calculated as follows;

Average percentage purity = (100.19 + 100.19 + 99.62)/ 3

= 100.00%

Uniformity of weight of Piroxicam capsules

Table U1 Uniformity of Weight, Ernest chemist Ltd

Piroxicam Capsules 20mg					
Name of Manufacturer: Ernest Chemist Ltd, Ghana					
Batch number: 0508J					
Weight of 20 capsules: 5.2602g					
Average weight: 0.2630g					
Number	**Wt of capsule**	**Wt of shell**	**Wt of content**	**Deviation**	**% deviation**
1	0.3223	0.0620	0.2603	-0.0027	1.0266
2	0.3148	0.0626	0.2522	-0.0108	4.1065
3	0.3350	0.0607	0.2743	0.0113	4.2966
4	0.3239	0.0633	0.2606	-0.0024	0.9125
5	0.3266	0.0630	0.2636	0.0006	0.2281
6	0.3309	0.0639	0.2670	0.0040	1.5209
7	0.3204	0.0632	0.2572	-0.0058	2.2053
8	0.3248	0.0644	0.2604	-0.0026	0.9886
9	0.3231	0.0619	0.2612	-0.0018	0.6844
10	0.3263	0.0618	0.2645	0.0015	0.5703
11	0.3294	0.0619	0.2675	0.0045	1.7110
12	0.3158	0.0642	0.2516	-0.0114	4.3346
13	0.3232	0.0626	0.2606	-0.0024	0.9125
14	0.3236	0.0613	0.2623	-0.0007	0.2662
15	0.3354	0.0620	0.2734	0.0104	3.9544
16	0.3218	0.0625	0.2593	-0.0037	1.4068
17	0.3310	0.0636	0.2674	0.0044	1.6730
18	0.3248	0.0629	0.2619	-0.0011	0.4183
19	0.3291	0.0624	0.2667	0.0037	1.4068
20	0.3321	0.0639	0.2682	0.0052	1.9772

Table U2 Uniformity of weight, Kinapharma Ltd, Ghana

Piroxicam Capsule 20mg					
Name of manufacturer: Kinapharma Ltd, Ghana					
Batch No: 10012					
Weight of 20 capsules: 5.537g					
Average weight: 0.2772g					
Number	**Wt of capsule**	**Wt of shell**	**Wt of content**	**Deviation**	**%Deviation**
1	0.3396	0.0627	0.2769	-0.0003	0.1082
2	0.3466	0.0622	0.2844	0.0072	2.5974
3	0.3434	0.0632	0.2802	0.0030	1.0082
4	0.3376	0.0623	0.2753	-0.0019	0.6854
5	0.3400	0.0616	0.2781	0.0009	0.3247
6	0.3315	0.0619	0.2696	-0.0076	2.7417
7	0.3340	0.0620	0.2695	-0.0077	2.7778
8	0.3456	0.0635	0.2821	0.0049	1.7677
9	0.3397	0.0615	0.2782	0.0010	0.3608
10	0.3435	0.0639	0.2796	0.0024	0.8658
11	0.3406	0.0624	0.2782	0.0010	0.3608
12	0.3391	0.0637	0.2754	-0.0018	0.6494
13	0.3362	0.0614	0.2748	-0.0024	0.8658
14	0.3368	0.0635	0.2733	-0.0039	1.4069
15	0.3334	0.0620	0.2714	-0.0058	2.0924
16	0.3421	0.0612	0.2809	0.0037	1.3348
17	0.3448	0.0619	0.2829	0.0057	0.1580
18	0.3395	0.0626	0.2769	-0.0003	0.1082
19	0.3423	0.0631	0.2792	0.0020	0.7215
20	0.3393	0.0628	0.2765	-0.0007	0.2525

Table U3 Uniformity of Weight, Letap Pharmaceutical, Ghana

Piroxicam Capsule 20mg					
Name of manufacturer: Letap Pharmaceutical Ltd, Ghana					
Batch no:0210131					
Weight of 20 capsules: 4.0532g					
Average weight: 0.2027g					
Number	**Wt of capsule**	**Wt of shell**	**Wt of content**	**Deviation**	**%Deviation**
1	0.2707	0.0637	0.2070	0.0043	2.1214
2	0.2735	0.0647	0.2088	0.0061	3.0094
3	0.2769	0.0571	0.2198	0.0171	8.4361
4	0.2601	0.0619	0.1982	-0.0045	2.2200
5	0.2643	0.0625	0.2018	-0.0009	0.4440
6	0.2543	0.0618	0.1925	-0.0102	5.0321
7	0.2581	0.0618	0.1963	-0.0064	3.1574
8	0.2520	0.0634	0.1886	-0.0141	6.9561
9	0.2626	0.0648	0.1978	-0.0049	2.4174
10	0.2679	0.0642	0.2037	0.0010	0.4933
11	0.2639	0.0660	0.1979	-0.0048	2.3680
12	0.2631	0.0617	0.2014	-0.0013	0.6413
13	0.2688	0.0641	0.2047	0.0020	0.9867
14	0.2666	0.0641	0.2033	0.0006	0.2600
15	0.2649	0.0638	0.2011	-0.0016	0.7893
16	0.2554	0.0624	0.1930	-0.0097	4.7854
17	0.2500	0.0587	0.1913	-0.0114	5.6241
18	0.2752	0.0651	0.2101	0.0074	3.6507
19	0.2835	0.0631	0.2004	-0.0023	1.1347
20	0.2806	0.0650	0.2156	0.0129	6.3641

Table U4 Uniformity of Weight, Luex Pharmaceuticals

Piroxicam capsules 20mg					
Name of Manufacturer: Luex Pharmaceuticals					
Batch no: 100428					
Weight of 20 capsules: 3.8287g					
Average weight: 0.1914g					
Number	**Wt of capsule**	**Wt of shell**	**Wt of content**	**Deviation**	**%Deviation**
1	0.2599	0.0635	0.1964	0.0050	2.6123
2	0.2532	0.0602	0.1912	-0.0002	0.1045
3	0.2593	0.0658	0.1935	0.0021	1.0972
4	0.2609	0.0664	0.1945	0.0031	1.6196
5	0.2437	0.0666	0.1995	0.0081	4.2319
6	0.2546	0.0674	0.1872	-0.0042	2.1944
7	0.2574	0.0655	0.1919	0.0005	0.2612
8	0.2724	0.0666	0.2058	0.0144	7.5235
9	0.2622	0.0657	0.1965	0.0051	2.6646
10	0.2500	0.0656	0.1844	-0.0070	3.6573
11	0.2465	0.0640	0.1825	-0.0089	4.6499
12	0.2653	0.0652	0.2001	0.0087	4.5454
13	0.2486	0.0662	0.1824	-0.0090	4.7022
14	0.2534	0.0678	0.1856	-0.0058	3.0303
15	0.2591	0.0637	0.1954	0.0040	2.0899
16	0.2557	0.0648	0.1909	-0.0005	0.2612
17	0.2481	0.0606	0.1875	-0.0039	2.0376
18	0.2515	0.0659	0.1856	-0.0058	3.0303
19	0.2612	0.0664	0.1948	0.0034	1.7764
20	0.2490	0.5660	0.1995	0.0081	4.2320

Uniformity of weight of Metronidazole tablets

Table U5 Uniformity of Weight, Ernest Chemist Limited, Ghana

Metronidazole Tablet 200mg			
Name of Manufacturer: Ernest Chemist Limited, Ghana			
Batch number: 0701L			
Weight of 20 Tablets: 11.823g			
Average weight: 0.5912g			
NUMBER	**WEIGHT OF TABLETS (g)**	**DEVIATION**	**% DEVIATION**
1	0.5909	-0.0003	0.0507
2	0.5957	0.0045	0.7612
3	0.5922	0.0010	0.1691
4	0.5924	0.0012	0.2030
5	0.5813	-0.0099	1.6746
6	0.5879	-0.0033	0.5582
7	0.5916	0.0004	0.0677
8	0.5855	-0.0057	0.9641
9	0.5944	0.0032	0.5413
10	0.5916	0.0004	0.0677
11	0.5908	-0.0004	0.0677
12	0.5911	-0.0001	0.0169
13	0.5906	-0.0006	0.1015
14	0.5930	0.0018	0.3045
15	0.5896	-0.0016	0.2706
16	0.5860	-0.0052	0.8796
17	0.5916	0.0004	0.0677
18	0.5975	0.0063	1.0656
19	0.5960	0.0048	0.8119
20	0.5933	0.0021	0.3552

Table U6 Uniformity of Weight, M & G Pharmaceuticals Limited, Ghana

Metronidazole Tablets 200mg			
Name of Manufacturer: M & G Pharmaceutical Limited, Ghana			
Batch Number: M2119			
Weight of 20 Tablets: 10.1284g			
Average weight: 0.5064g			
NUMBER	**WEIGHT OF TABLETS (g)**	**DEVIATION**	**% DEVIATION**
1	0.5013	-0.0051	1.0071
2	0.5222	0.0158	3.1201
3	0.5150	0.0086	1.6983
4	0.5091	0.0027	0.5332
5	0.4917	-0.0147	2.9028
6	0.5062	-0.0002	0.0395
7	0.5027	-0.0037	0.7306
8	0.5122	0.0058	1.1453
9	0.4954	-0.0110	2.1722
10	0.4961	-0.0103	2.0340
11	0.5011	-0.0053	1.0466
12	0.4977	-0.0087	1.7180
13	0.5262	0.0196	3.9100
14	0.5292	0.0228	4.5024
15	0.4961	-0.0103	2.0340
16	0.5086	0.0022	0.4344
17	0.4922	-0.0142	2.8041
18	0.5000	-0.0064	1.2638
19	0.5055	-0.0009	0.1777
20	0.5199	0.0135	2.6659

Table U7 Uniformity of Weight, Letap Pharmaceuticals Limited, Ghana

Metronidazole Tablets 200mg			
Name of Manufacturer: Letap Pharmaceuticals Limited, Ghana			
Batch Number: 0740881			
Weight of 20 Tablets: 10.0404g			
Average weight: 0.5020g			
NUMBER	**WEIGHT OF TABLETS (g)**	**DEVIATION**	**% DEVIATION**
1	0.5185	0.0165	3.2869
2	0.5154	0.0134	2.6693
3	0.5184	0.0164	3.2669
4	0.4867	-0.0153	3.0478
5	0.4914	-0.0106	2.1115
6	0.5149	0.0129	2.5697
7	0.5019	-0.0001	0.0199
8	0.4942	-0.0078	0.3916
9	0.4821	-0.0199	3.9641
10	0.5018	-0.0002	0.0398
11	0.5021	0.0001	0.0199
12	0.4964	-0.0056	1.1155
13	0.5005	-0.0015	0.2988
14	0.5003	-0.0017	0.3386
15	0.4965	-0.0055	1.0956
16	0.5074	0.0054	1.0757
17	0.5038	0.0018	0.3586
18	0.5077	0.0057	1.1355
19	0.4948	-0.0072	1.4343
20	0.5056	0.0036	0.7171

Table U8 Uniformity of Weight, Maxheal Laboratories Ltd

Metronidazole Tablets 200mg			
Name of Manufacturer: Maxheal Laboratories Ltd			
Batch Number: XT015			
Weight of 20 Tablets: 12.7893g			
Average weight: 0.6396g			
NUMBER	**WEIGHT OF TABLETS (g)**	**DEVIATION**	**% DEVIATION**
1	0.6350	-0.0046	0.7192
2	0.6489	0.0093	1.4540
3	0.6449	0.0053	0.8286
4	0.6369	-0.0027	0.4221
5	0.6429	0.0033	0.5159
6	0.6401	0.0005	0.0782
7	0.6352	-0.0044	0.6879
8	0.6438	0.0042	0.6567
9	0.6406	0.0010	0.1563
10	0.6442	0.0046	0.7192
11	0.6297	-0.0099	1.5478
12	0.6437	0.0041	0.6410
13	0.6345	-0.0051	0.7974
14	0.6316	-0.0080	1.2508
15	0.6469	0.0073	1.1413
16	0.6368	-0.0028	0.4378
17	0.6372	-0.0024	0.3752
18	0.6354	-0.0042	0.6567
19	0.6424	0.0028	0.4378
20	0.6413	0.0017	0.2658

Table RTP Retention time for Piroxicam and its surrogates

Sample	Mean Retention time
Piroxicam	6.83±0.065
Metronidazole	4.07±0.058
Metformin	3.42±0.049
Ascorbic acid	2.56±0.055

Table RPM Retention time for Metronidazole and its surrogates

Sample	Mean Retention time
Metronidazole	4.24 ± 0.014
Ascorbic acid	1.80 ± 0.012
Para aminophenol	2.82 ± 0.015

Table LDP Limit of detection and limit of quantification for Piroxicam and its surrogates

Sample	LOD (%$^{w}/_{v}$)	LOQ (%$^{w}/_{v}$)
Piroxicam	0.000159	0.00048
Metronidazole	0.000077	0.00023
Metformin	0.000074	0.00022
Ascorbic acid	0.000064	0.00019

Table LDM Limit of detection and limit of quantification for Metronidazole and its surrogates

Sample	LOD (%$^{w}/_{v}$)	LOQ (%$^{w}/_{v}$)
Metronidazole	0.0000185	0.0000560
Ascorbic acid	0.000334	0.001032
Para aminophenol	0.0000295	0.0000891

Linearity

Linearity for Piroxicam and its surrogates

Table LPP Linearity for Piroxicam

Range of concentration: 0.0002 – 0.0060%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 3988.1x + 0.3674	0.9982
y = 3982.7x + 0.4234	0.9984
y = 3970.6x + 0.4015	0.9982

Table LPM Linearity for Metronidazole

Range of concentration: 0.0002 – 0.0060%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 5327.3x + 0.5156	0.9993
y = 5330.8x + 0.6034	0.9990
y = 5285.5x + 0.603	0.9979

Table LPF Linearity for Metformin

Range of concentration: 0.0002 – 0.0060%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 8956.5x – 0.3181	0.9996
y = 8949.6x – 0.2752	0.9996
y = 8992.6x – 0.303	0.9997

Table LPA Linearity for Ascorbic acid

Range of concentration: 0.0002 – 0.004%$^{w}/_{v}$	
Equation of the line	**Correlation coefficient, R^2**
y = 12558x + 1.3721	0.9988
y = 12751x + 1.6749	0.9984
y = 12556x + 1.2281	0.9993

Linearity for Metronidazole and its surrogates

Table LMM Linearity for Metronidazole

Range of concentration: 0.002 – 0.01%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 1004.5x + 0.243	0.9995
y = 994.5x + 0.275	0.9993
y = 1009x + 0.212	0.9996

Table LMA Linearity for Ascorbic acid

Range of concentration: 0.002 – 0.01%$^{w}/_{v}$	
Equation of line	**Correlation coefficient, R^2**
y = 1889x – 1.298	0.9955
y = 1875.5x – 1.219	0.9963
y = 1894x – 1.25	0.9953

Table LMP Linearity for Para aminophenol

Range of concentration: 0.002 – 0.01%$^{w}/_{v}$	
Equation of the line	**Correlation coefficient, R^2**
y = 809x + 0.118	0.9989
y = 811x + 0.194	0.9975
y = 830x + 0.016	0.9977

Specificity

Table SSP Piroxicam

Sample	**Retention time**
Piroxicam pure sample	6.83±0.065
Piroxicam capsules	6.84±0.016

Table SSM Metronidazole

Sample	**Retention time**
Metronidazole pure sample	4.24 ± 0.014
Metronidazole tablets	4.26 ± 0.006

Precision (repeatability)

Piroxicam and its surrogates

Table PPP Piroxicam

Relative Standard Deviation of Pure Piroxicam, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.004	16.82	16.80	16.82	16.71	16.90	16.85	16.82	0.0628	0.3734
0.002	8.32	8.46	8.32	8.38	8.42	8.40	8.38	0.0557	0.6644
0.001	4.95	4.93	4.93	4.89	4.81	4.99	4.92	0.0615	1.2508

Table PPM Metronidazole

Relative Standard Deviation of Pure Metronidazole, Number of injections = 6									
Concentration (%w/v)	Peak Area						Mean Peak Area	Standard Deviation	RSD (%)
	1	2	3	4	5	6			
0.004	22.16	22.20	22.16	22.30	22.24	22.28	22.22	0.0599	0.2696
0.002	11.24	11.04	11.44	11.35	11.25	11.19	11.25	0.1370	1.2178
0.001	6.32	6.66	6.39	6.45	6.50	6.55	6.48	0.1202	1.8554

Table PPF Metformin

Relative Standard Deviation of Pure Metformin, Number of injections = 6									
Concentration (%w/v)	Peak Area						Mean Peak Area	Standard Deviation	RSD (%)
	1	2	3	4	5	6			
0.004	35.99	35.68	35.98	35.71	35.78	35.84	35.83	0.1324	0.3695
0.002	17.46	17.16	17.65	17.41	17.32	17.50	17.42	0.1665	0.9559
0.001	8.55	8.56	8.75	8.61	8.67	8.70	8.64	0.0800	0.9259

Table PPA Ascorbic acid

Relative Standard Deviation of Pure Ascorbic Acid, Number of injections = 6									
Concentration (%w/v)	Peak Area						Mean Peak Area	Standard Deviation	RSD (%)
	1	2	3	4	5	6			
0.004	51.12	52.08	51.10	52.48	51.39	51.95	51.69	0.5669	1.0969
0.002	27.45	28.46	26.95	27.91	28.14	27.65	27.76	0.5331	1.9220
0.001	14.00	14.37	14.01	13.49	14.87	13.38	14.02	0.5543	3.9536

Metronidazole and its surrogates

Table PMM Metronidazole

Relative Standard Deviation of Pure Metronidazole, Number of injections = 6									
Concentration (%w/v)	Peak Area						Mean Peak Area	Standard Deviation	RSD (%)
	1	2	3	4	5	6			
0.010	10.25	10.31	9.85	10.01	9.89	10.18	10.08	0.1927	1.9117
0.008	8.28	8.36	8.27	8.27	8.29	8.30	8.30	0.0339	0.4087
0.006	6.19	6.20	6.19	6.15	6.23	6.24	6.20	0.0322	0.5194

Table PMA Ascorbic acid

Relative Standard Deviation of Pure Ascorbic Acid, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.010	18.15	18.04	17.89	18.26	18.20	18.18	18.12	0.1340	0.7363
0.008	13.64	13.48	13.60	13.51	13.52	13.66	13.57	0.0749	0.5514
0.006	9.79	9.68	9.78	9.64	9.74	9.80	9.74	0.0652	0.6695

Table PMP Para aminophenol

Relative Standard Deviation of Pure Para aminophenol, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.010	8.15	8.34	8.21	8.25	8.28	8.24	8.25	0.0641	0.7774
0.008	6.68	6.58	6.75	6.72	6.67	6.64	6.67	0.0599	0.8976
0.006	5.01	5.22	5.14	5.18	5.10	5.15	5.13	0.0723	1.4085

Determination of the constant K for Piroxicam capsules

Table PK 1 Determination of K values for Piroxicam pure sample using Metronidazole as the surrogate standard. Percentage Purity of Metronidazole = 100.49%				
Concentration of metronidazole	**Peak Area of Metronidazole**	**Concentration of Piroxicam**	**Peak Area of Piroxicam**	**K value**
0.0020	2.59	0.0020	5.01	1.9344
0.0016	2.14	0.0020	5.22	1.9314
0.0016	1.99	0.0016	3.87	1.9447
0.0020	2.89	0.0016	4.58	1.9556
0.0020	2.60	0.0020	4.99	1.9392
Average K value = 1.9411± 0.004				

Table PK 2 Determination of K values for Piroxicam pure sample using Metformin as the surrogate standard. Percentage Purity of Metformin = 100.32%				
Concentration of Metformin	**Peak Area of Metformin**	**Concentration of Piroxicam**	**Peak Area of Piroxicam**	**K value**
0.0020	4.62	0.0020	7.52	1.6266
0.0016	4.19	0.0020	7.95	1.6286
0.0016	3.70	0.0016	6.36	1.6061
0.0020	4.67	0.0020	7.62	1.6334
0.0020	5.24	0.0016	6.08	1.6051
Average K value = 1.6200 ± 0.006				

Table PK 3 Determination of K values for Piroxicam pure sample using Ascorbic acid as the surrogate standard. Percentage Purity of Ascorbic acid = 100.00%				
Concentration of Ascorbic acid	**Peak Area of Ascorbic acid**	**Concentration of Piroxicam**	**Peak Area of Piroxicam**	**K value**
0.002	11.55	0.0020	5.40	0.4675
0.0016	9.25	0.0016	4.33	0.4649
0.0018	10.20	0.0016	3.99	0.4400
0.0016	10.45	0.0020	6.12	0.4685
0.0018	11.00	0.0020	5.42	0.4435
Average K value = 0.4569 ± 0.006				

Determination of the constant K, for Metronidazole tablets

Table MK 1 Determination of K values for Metronidazole pure sample using Ascorbic acid as the surrogate standard. Percentage Purity of Ascorbic acid = 100.00%				
Concentration of Ascorbic acid	**Peak Area of Ascorbic acid**	**Peak Area of Metronidazole**	**Concentration of Metronidazole**	**K value**
0.01	12.74	8.75	0.01	0.6864
0.005	8.93	12.11	0.01	0.6784
0.01	16.83	5.62	0.005	0.6674
0.005	6.37	4.34	0.005	0.6808
0.01	12.92	8.64	0.01	0.6685
Average K = 0.6763 ± 0.004				

Table MK 2 Determination of K values for Metronidazole pure sample using Para aminophenol as the surrogate standard.				
Concentration of Paraaminophenol	**Peak Area of Paraaminophenol**	**Peak Area of Metronidazole**	**Concentration of Metronidazole**	**K value**
0.01	7.28	7.95	0.01	1.0928
0.005	5.39	12.75	0.01	1.1838
0.005	3.66	4.07	0.005	1.1100
0.01	9.63	5.52	0.005	1.1250
0.01	7.37	8.13	0.01	1.1030
Average K = 1.1229 ± 0.016				

Determination of Percentage Content of Piroxicam in Piroxicam capsules

Table PPE 1 Percentage content of Piroxicam in Piroxicam capsules using Metronidazole as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2630g					
Name of Manufacturer: Ernest Chemist Limited, Ghana					
Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
8.46	0.006	0.09486	16.42171	0.005777	96.28
7.13	0.005	0.06625	13.84004	0.004787	95.74
5.66	0.004	0.04232	10.98663	0.003852	96.30
2.83	0.002	0.01056	5.493313	0.001922	96.12
1.42	0.001	0.00026	2.756362	0.009542	95.42

Table PPE 2 Percentage content of Piroxicam in Piroxicam capsules using Metformin as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2630g					
Name of Manufacturer: Ernest Chemist Limited, Ghana					
Peak Area of Metformin (As)	Concentration of Metformin (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
14.91	0.006	0.13770	24.1542	0.005700	95.01
12.75	0.005	0.09705	20.6550	0.004699	93.97
10.00	0.004	0.06128	16.2000	0.003783	94.57
4.96	0.002	0.01532	8.05140	0.001903	95.14
2.49	0.001	0.00382	4.03380	0.000947	94.70

Table PPE 3 Percentage content of Piroxicam in Piroxicam capsules using Ascorbic acid as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2630g					
Name of Manufacturer: Ernest Chemist Limited, Ghana					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
39.31	0.006	0.10296	17.96074	0.0057325	95.54
33.90	0.005	0.07435	15.48891	0.0048002	96.00
26.95	0.004	0.04752	12.31346	0.0038592	96.48
13.61	0.002	0.01198	6.218410	0.0019265	96.32
6.79	0.001	0.00298	3.102351	0.0009606	96.06

Table PPK 1 Percentage content of Piroxicam in Piroxicam capsules using Metronidazole as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2772g					
Name of Manufacturer: Kinapharma Limited, Ghana					
Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
8.48	0.006	0.09696	16.46053	0.005890	98.17
7.86	0.005	0.07570	15.25705	0.004975	99.50
6.29	0.004	0.04872	12.20952	0.003990	99.75
3.37	0.002	0.01288	6.54150	0.001970	98.44
1.58	0.001	0.00302	3.06694	0.000985	98.47

Table PPK 2 Percentage content of Piroxicam in Piroxicam capsules using Metformin as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2772g					
Name of Manufacturer: Kinapharma Limited, Ghana					
Peak Area of Metformin (As)	Concetration of Metformin (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
16.28	0.006	0.15168	26.3736	0.005751	95.85
12.65	0.005	0.10075	20.4930	0.004916	98.34
10.62	0.004	0.06596	17.2044	0.003834	95.84
5.23	0.002	0.01668	8.47260	0.001969	98.43
1.62	0.001	0.00423	4.42260	0.000956	95.65

Table PPK 3 Percentage content of Piroxicam in Piroxicam capsules using Ascorbic acid as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2772g					
Name of Manufacturer: Kinapharma Limited, Ghana					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
36.97	0.006	0.09984	16.8916	0.005911	98.51
32.82	0.005	0.07490	14.9955	0.004998	99.89
26.00	0.004	0.04700	11.8794	0.003956	98.91
12.99	0.002	0.01176	5.9351	0.001981	99.07
6.53	0.001	0.00301	2.98356	0.001009	100.89

Table PPL 1 Percentage content of Piroxicam in Piroxicam capsules using Metronidazole as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2027g					
Name of Manufacturer: Letap Pharmaceuticals, Ghana					
Peak Area of Metronidazole (As)	Concentration of A Metronidazole (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
7.14	0.006	0.08454	13.8595	0.0060998	101.66
6.78	0.005	0.06650	13.1607	0.0050529	101.06
5.39	0.004	0.04292	10.4625	0.0041023	102.56
2.68	0.002	0.01066	5.20215	0.0020492	102.46
1.36	0.001	0.00267	2.63989	0.0010114	101.14

Table PPL 2 Percentage content of Piroxicam in Piroxicam capsules using Metformin as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2027g					
Name of Manufacturer: Letap Pharmaceuticals, Ghana					
Peak Area of Metformin (As)	Concentration of A Metformin (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
15.89	0.006	0.15630	25.7418	0.0060718	101.20
12.86	0.005	0.10760	20.8332	0.0051648	103.30
10.49	0.004	0.07004	16.9938	0.0041215	103.04
5.31	0.002	0.01738	8.60220	0.0020204	101.02
2.76	0.001	0.00455	4.47120	0.0010176	101.76

Table PPL 3 Percentage content of Piroxicam in Piroxicam capsules using Ascorbic acid as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.2027g					
Name of Manufacturer: Letap Pharmaceuticals, Ghana					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
33.95	0.006	0.09348	15.51176	0.0060264	100.44
27.89	0.005	0.06375	12.74294	0.0050028	100.06
23.57	0.004	0.04384	10.76913	0.0040610	101.75
11.86	0.002	0.01086	5.418834	0.0020412	100.21
5.89	0.001	0.00275	2.691141	0.0010219	102.19

Table PPU 1 Percentage content of Piroxicam in Piroxicam capsules using Metronidazole as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.1914g					
Name of Manufacturer: Luex Pharmaceutical					
Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
7.20	0.006	0.0822	13.9759	0.005882	98.03
6.05	0.005	0.0574	11.7437	0.004888	97.75
4.81	0.004	0.0365	9.3367	0.003907	97.68
2.39	0.002	0.0092	4.6392	0.001974	98.72
1.21	0.001	0.0023	2.3487	0.000979	97.93

Table PPU 2 Percentage content of Piroxicam in Piroxicam capsules using Metformin as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.1914g					
Name of Manufacturer: Luex Pharmaceuticals					
Peak Area of Metformin (As)	Concentration of Metformin (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
15.48	0.006	0.14754	25.0776	0.005883	98.06
13.55	0.005	0.10755	21.9510	0.004899	97.99
10.81	0.004	0.06880	17.5122	0.003929	98.22
5.38	0.002	0.01714	8.71560	0.001967	98.33
2.75	0.001	0.00436	4.45500	0.000979	97.87

Table PPU 3 Percentage content of Piroxicam in Piroxicam capsules using Ascorbic acid as the surrogate standard.					
Capsule: Piroxicam, 20mg; Average weight = 0.1914g					
Name of Manufacturer: Luex Pharmaceuticals					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	Aa x Cs / K x As	Percentage content
35.71	0.006	0.09594	16.315899	0.005880	98.00
28.95	0.005	0.06515	13.227255	0.004926	98.50
22.98	0.004	0.04184	10.499562	0.003985	99.62
11.89	0.002	0.01082	5.4325410	0.001992	99.59
5.99	0.001	0.00266	2.7368310	0.000972	97.19

Determination of Percentage Content of Metronidazole in Metronidazole tablets

Table PME 1 Percentage content of Metronidazole in Metronidazole tablets using Ascorbic acid as the surrogate standard.					
Tablets: Metronidazole, 200mg; Average weight = 0.5912g					
Name of Manufacturer: Ernest Chemist Limited, Ghana					
Peak Area of Ascorbic acid (As)	**Concentration of Ascorbic acid (Cs)**	**Aa x Cs**	**K x As**	**(Aa x Cs)/ K x As**	**Percentage content**
11.98	0.010	0.0801	8.102074	0.009886	98.86
9.78	0.008	0.05128	6.61421	0.007753	96.91
6.17	0.005	0.02035	4.17277	0.004877	97.54
2.40	0.002	0.00318	1.62312	0.001959	97.95
6.23	0.005	0.0208	4.2472	0.0048974	97.95

Table PME 2 Percentage content of Metronidazole in Metronidazole tablets using para aminophenol as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.5912g					
Name of Manufacturer: Ernest Chemist Limited, Ghana					
Peak Area of Paraaminphenol (As)	**Concentration of Paraaminophenol (Cs)**	**Aa x Cs**	**K x As**	**(Aa x Cs)/ K x As**	**Percent. content**
7.22	0.01	0.0799	8.10734	0.009855	98.55
5.81	0.008	0.05136	6.52405	0.007872	98.40
3.65	0.005	0.0203	4.0986	0.004953	99.06
1.48	0.002	0.00328	1.6619	0.001974	98.68
3.68	0.005	0.02015	4.1323	0.004876	97.53

Table PML 1 Percentage content of Metronidazole in Metronidazole tablets using Ascorbic acid as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.5020g					
Name of Manufacturer: Letap Pharmaceuticals, Ghana					
Peak Area of Ascorbic acid (As)	**Concentration of Ascorbic acid (Cs)**	**Aa x Cs**	**K x As**	**(Aa x Cs)/ K x As**	**Percent. content**
11.85	0.010	0.0839	8.0142	0.010469	104.69
9.49	0.008	0.05336	6.418	0.008314	103.93
6.45	0.005	0.0226	4.3624	0.0051806	103.61
2.43	0.002	0.00342	1.6434	0.0020811	104.05
6.68	0.005	0.0236	4.5177	0.005224	104.48

Table PML 2 Percentage content of Metronidazole in Metronidazole tablets using Paraaminophenol as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.5020g					
Name of Manufacturer: Letap Pharmaceuticals, Ghana					
Peak Area of Paraaminphenol (As)	Concentration of Paraaminophenol (Cs)	Aa x Cs	K x As	(Aa x Cs)/ K x As	Percent. content
7.28	0.010	0.0849	8.1747	0.010386	103.86
5.84	0.008	0.05448	6.5577	0.0083077	103.85
3.68	0.005	0.0216	4.1323	0.005227	104.54
1.51	0.002	0.00354	1.6956	0.0020878	104.38
3.54	0.005	0.02085	3.9751	0.005245	104.90

Table PMM 1 Percentage content of Metronidazole in Metronidazole tablets using Ascorbic acid as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.5064g					
Name of Manufacturer: M & G Pharmaceuticals, Ghana					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	(Aa x Cs)/ K x As	Percent. content
11.86	0.010	0.0770	7.89918	0.009748	97.48
9.38	0.008	0.4928	6.34369	0.00768	97.10
5.83	0.005	0.01945	3.94283	0.00493	98.66
2.33	0.002	0.00308	1.57578	0.001955	97.73
6.29	0.005	0.02105	4.253927	0.00495	98.97

Table PMM 2 Percentage content of Metronidazole in Metronidazole tablets using paraaminophenol as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.5064g					
Name of Manufacturer: M & G Pharmaceuticals, Ghana					
Peak Area of Paraaminphenol (As)	Concentration of Paraaminophenol (Cs)	Aa x Cs	K x As	(Aa x Cs)/ K x As	Percent. content
7.87	0.010	0.08610	8.837223	0.009743	97.43
6.35	0.008	0.05512	7.1304	0.007730	96.63
3.95	0.005	0.02165	4.435455	0.004881	97.62
1.58	0.002	0.00344	1.774182	0.001939	96.95
4.22	0.005	0.02340	4.738638	0.004938	98.76

Table PMX 1 Percentage content of Metronidazole in Metronidazole tablets using Ascorbic acid as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.6396g					
Name of Manufacturer: Maxheal Laboratories Limited, India					
Peak Area of Ascorbic acid (As)	Concentration of Ascorbic acid (Cs)	Aa x Cs	K x As	(Aa x Cs)/ K x As	Percent. content
12.48	0.010	0.0882	8.440	0.010450	104.50
9.96	0.008	0.05688	6.7356	0.008444	105.56
6.89	0.005	0.02425	4.6597	0.005204	104.08
2.12	0.002	0.0298	1.4338	0.002079	103.92
6.83	0.005	0.0237	4.619	0.00513	102.62

Table PMX 2 Percentage content of Metronidazole in Metronidazole tablets using Paraaminophenol as the surrogate standard.					
Tablet: Tablets: Metronidazole, 200mg; Average weight = 0.6396g					
Name of Manufacturer: Maxheal Laboratories Limited, India					
Peak Area of Paraaminophenol (As)	Concentration of Paraaminophenol (As)	Aa x Cs	K x As	(Aa x Cs)/ K x As	Percent. Content
8.49	0.010	0.0993	9.5334	0.0104160	104.16
6.50	0.008	0.0612	7.2989	0.0083849	104.81
4.35	0.005	0.0257	4.8846	0.0052614	105.22
1.70	0.002	0.0040	1.9089	0.0020849	104.25
4.59	0.005	0.0266	5.1541	0.0051512	103.02